

OXIDATION PRODUCTS OF SYNTHETIC POLYUNSATURATED FATTY ACIDS

A Thesis submitted for the degree of

Doctor of Philosophy

of

The Australian National University



Research School Of Chemistry

by

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BSc (Hons)

September 1997

Acknowledgements

Many people have contributed to the research work that is the basis of this thesis. I am particularly grateful to Dr. Chris Eason for your excellent supervision and support throughout the duration of my project. To Prof. G. J. Poulis, Prof. T. J. Fennell and Dr. Debbie Ralston thank you for your additional supervision and advice, particularly in the early stages of the project. The funding for this work was provided by Repatriate Ltd via the Adelaide Women's and Children's Hospital. I am indebted to the people of both organisations for their investment in the research project of which my work is part.

Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or other institute of higher learning, except where due acknowledgement is made in the text.

Tom Robertson

Thomas Robertson

September 1997

Acknowledgements

Many people have contributed to the research contained in the following pages. Thanks especially to Dr Chris Easton for your excellent supervision and support for the duration of my project. To Prof. Alf Poulos, Prof. Tony Ferrante and Dr Debbie Rathjen thank you for your additional supervision and advice, particularly in the early stages of the project. The funding for this work was provided by Peptech Ltd *via* the Adelaide Women's and Children's Hospital. I am indebted to the people of both organisations for their investment in the research project of which my work is part.

To Dr Mick Pitt, a thousand thanks for providing most of the synthetic fatty acids used throughout the project and in addition thank you for your endless enthusiasm, encouragement and humour whilst working with me. I would also like to thank the people from Peptech Ltd who provided the remaining synthetic fatty acids.

I am grateful for the excellent technical assistance I have received whilst studying at both the University of Adelaide and the Australian National University. In particular, thank you to Carl Braybrook at the ANU Research School of Chemistry for your assistance in running the mass spectrometry experiments.

To my research group, moving to Canberra wasn't easy and the companionship you have provided has been invaluable. I've enjoyed working in the group and I hope the unity and selflessness within it continues in the years to come. To the remaining staff and students in both Adelaide and Canberra, thank you for your help, encouragement and friendship over the past few years.

To my family thanks for the support and encouragement you have given me, particularly in helping me choose to pursue an occupation that I find stimulating. Nicole, congratulations and thank you for your continuing friendship and faith in me.

Abstract

The six hydroxyeicosatetraenoic acid (HETE) regioisomers **48-53** have been prepared from arachidonic acid **3** by thin film autoxidation. Following separation of the isomers **48-53** by HPLC, tandem electrospray mass spectroscopy was used to identify each regioisomer on the basis of the characteristic daughter ions.

An HPLC assay was used to monitor the consumption of a mixture of arachidonic acid **3** and one of the synthetic polyunsaturated fatty acids (PUFAs) **83**, **84** and **86** with 15-lipoxygenase (LO) or 5-LO. The assay confirmed that in the presence of the known substrate arachidonic acid **3** the synthetic PUFAs **84** and **86** are substrates for both enzymes and the compound **83** is a substrate for 15-LO.

UV spectroscopy was used to monitor the formation of products containing a conjugated diene moiety during incubation of each of the PUFAs **83-89** with 5-LO or 15-LO. Conjugated diene only failed to form in the cases of the diacids **87** and **89** with 5-LO. In every other case, conjugated diene formed at varying maximum rates and yields based on the maximum rate of change and total change in absorbance, respectively.

The oxidation products formed by incubation of the PUFAs **83-89** with 15-LO were reduced with sodium borohydride and characterised by ^1H and ^{13}C nmr spectroscopy. ^1H nmr spectroscopy indicated that a *2E,4Z*-dienol system had formed in each case. By HPLC one major oxidation product was observed following oxidation of each of the synthetic PUFAs **83-89** using 15-LO. The synthetic PUFAs **83-86** and **88** were oxidised during incubation with 5-LO, a component of the reaction mixture containing a single oxidation product was isolated by HPLC and the presence of an oxidation product was confirmed in each case based on the presence of the appropriate molecular ion by mass spectrometric analysis.

The products **96-108** formed by oxidation of the PUFAs **83-89** with 15-LO or by oxidation of the PUFAs **83-86** and **88** with 5-LO and subsequent reduction with sodium borohydride were identified on the basis of characteristic daughter ions formed using tandem electrospray mass spectrometry. Oxidation of the synthetic PUFAs **83-89** by 15-LO took place at the (n-6) carbon. In addition to oxidation of an olefinic carbon at the (n-6) position, the sulfur of the β -thioether **87** was also oxidised during incubation with 15-LO to form a sulfoxide. Oxidation of the synthetic PUFAs **83-86** by 5-LO took place at the olefinic carbon closest to the carboxylic acid group. Based on the daughter ion mass spectrum, oxidation of the amide **88** by 5-LO did not occur at C-5' and instead occurred selectively at C-11'.

The alcohol **96** formed by oxidation of the β -oxa compound **84** with 15-LO was incubated with 5-LO and by UV spectroscopy a triene product had formed. The diol **109** derived from this product was identified by mass spectrometry. In a coincubation experiment with 5-LO, conjugated diene formation from arachidonic acid **3** was significantly inhibited in the presence of the alcohol **96** at a 5 μ M concentration and inhibited by over 90% at a 50 μ M concentration.

Abbreviations

The following abbreviations have been used in this thesis:

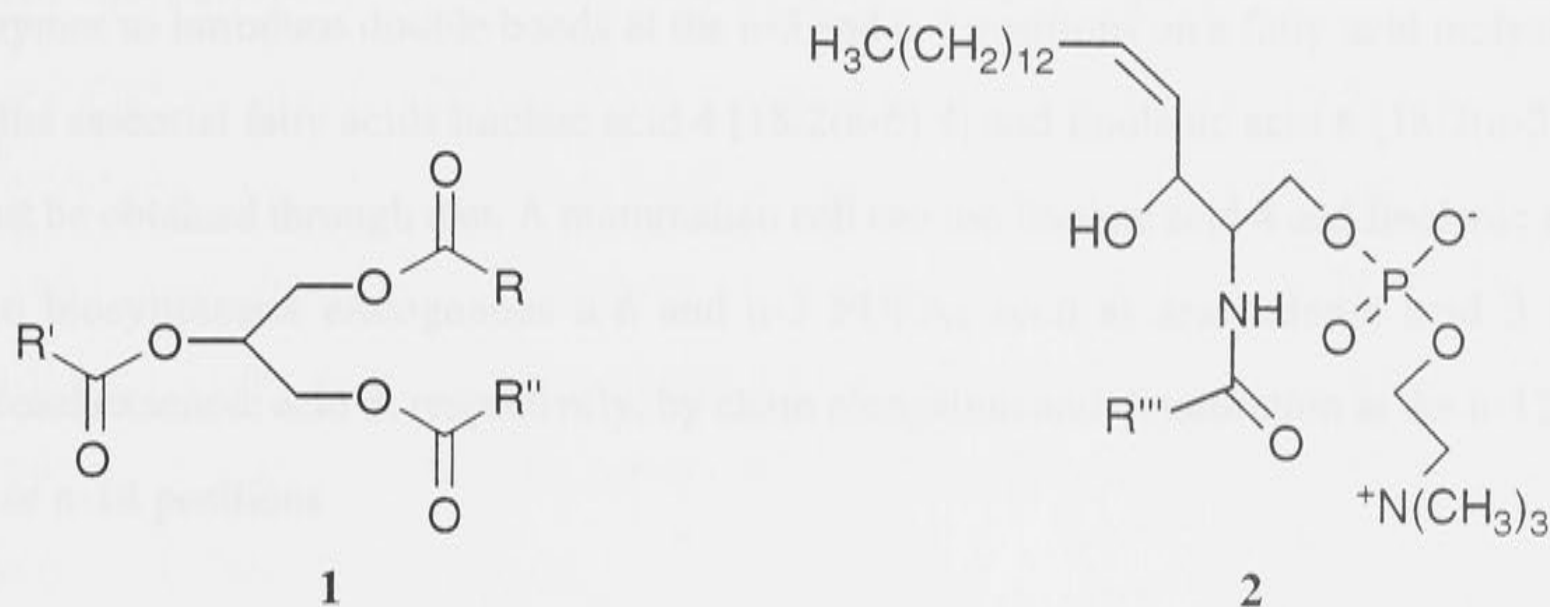
AIBN	azobis(isobutyronitrile)
ATP	adenosine triphosphate
BHT	butylated hydroxytoluene
CID	collision induced disociation
ϵ	extinction coefficient
FAB-MS/MS	fast atom bombardment tandem mass spectrometry
GC-MS	gas chromatography - mass spectrometry
HANA	hydroxyanandamide
HETE	hydroxyeicosatetraenoic acid
HPANA	hydroperoxyanandamide
HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high performance liquid chromatography
kETE	ketoecicosatetraenoic acid
K_m	Michaelis constant
IR	infra-red
$^3J_{HH}$	vicinal coupling constant
λ_{max}	maximum absorbance wavelength
LO	lipoxygenase
11,15-LO	cyclooxygenase
MeHETE	methyl hydroxyeicosatetraenoate
MeHPODE	methyl hydroperoxyoctadecadienoate
MeHODE	methyl hydroxyoctadecadienoate
MeHPOTE	methyl hydroperoxyoctadecatrienoate
MeHOTE	methyl hydroxyoctadecatrienoate
nmr	nuclear magnetic resonance
ODS	octadecylsilane
PML	polymorphonuclear leukocyte
ppm	parts per million
PUFA	polyunsaturated fatty acid
RBC	red blood cells
RBL	rat basophilic leukemia cell
R_t	retention time
TLC	thin layer chromatography
UV	ultra-violet
V_{max}	maximum rate
WCH	Women's and Children's Hospital

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Introduction

Fatty acids are components of lipids, one of the main classes of compounds common in biological systems. In nature they are most often found in the esterified form or linked to long chain bases as amides in compounds such as triglycerides **1** and sphingomyelins **2**. Fatty acids commonly have unbranched structure and an even number of carbons per molecule.^{1,2}



R, R', R'', R''' = alkyl / alkenyl chains

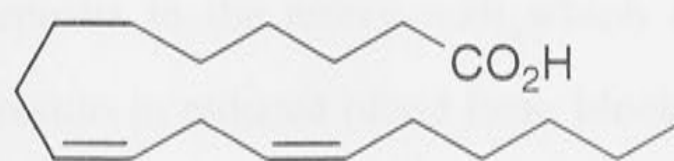
Polyunsaturated fatty acids (PUFAs) are a group of naturally occurring fatty acids distinguished by the presence of double bonds of Z-geometry within a methylene interrupted polyene system. In naturally occurring PUFAs the polyene system commences either 3, 6, or 9-carbons from the methyl terminus. The nomenclature of PUFAs includes trivial names, systematic names and a numeric system, such that the compound commonly known as arachidonic acid **3** is formally named (5Z,8Z,11Z,14Z)-eicosatetraenoic acid **3**, and using the numeric system the name is 20:4(n-6) **3**.¹⁻³ The name 20:4(n-6) **3** indicates the number of carbons followed by the number of double bonds in the molecule, and in parentheses is the number of carbons from the methyl terminus 'n' at which the methylene interrupted polyene system commences. So 20:4(n-6) **3** is a 20 carbon molecule with 4 double bonds and the methylene interrupted tetraene system commences 6 carbons from the methyl terminus.

**3**

Trivial: arachidonic acid

Formal: (5Z,8Z,11Z,14Z)-eicosatetraenoic acid

Numeric: 20:4(n-6)

**4**

linoleic acid

(9Z,12Z)-octadecadienoic acid

18:2(n-6)

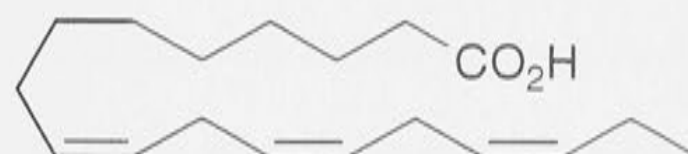
Fatty acids make up between 25-30% of the average human diet.³ Mammals lack the enzymes to introduce double bonds at the n-3 and n-6 positions on a fatty acid molecule, so the essential fatty acids linoleic acid **4** [18:2(n-6) **4**] and linolenic acid **6** [18:3(n-3) **6**] must be obtained through diet. A mammalian cell can use linoleic acid **4** and linolenic acid **6** to biosynthesise endogenous n-6 and n-3 PUFAs such as arachidonic acid **3** and docosahexaenoic acid **5**, respectively, by chain elongation and desaturation at the n-12, n-15 or n-18 positions.

**5**

Trivial: docosahexaenoic acid

Formal: (4Z,7Z,10Z,13Z,16Z,19Z)-
docosahexaenoic acid

Numeric: 22:6(n-3)

**6**

linolenic acid

(9Z,12Z,15Z)-octadecatrienoic acid

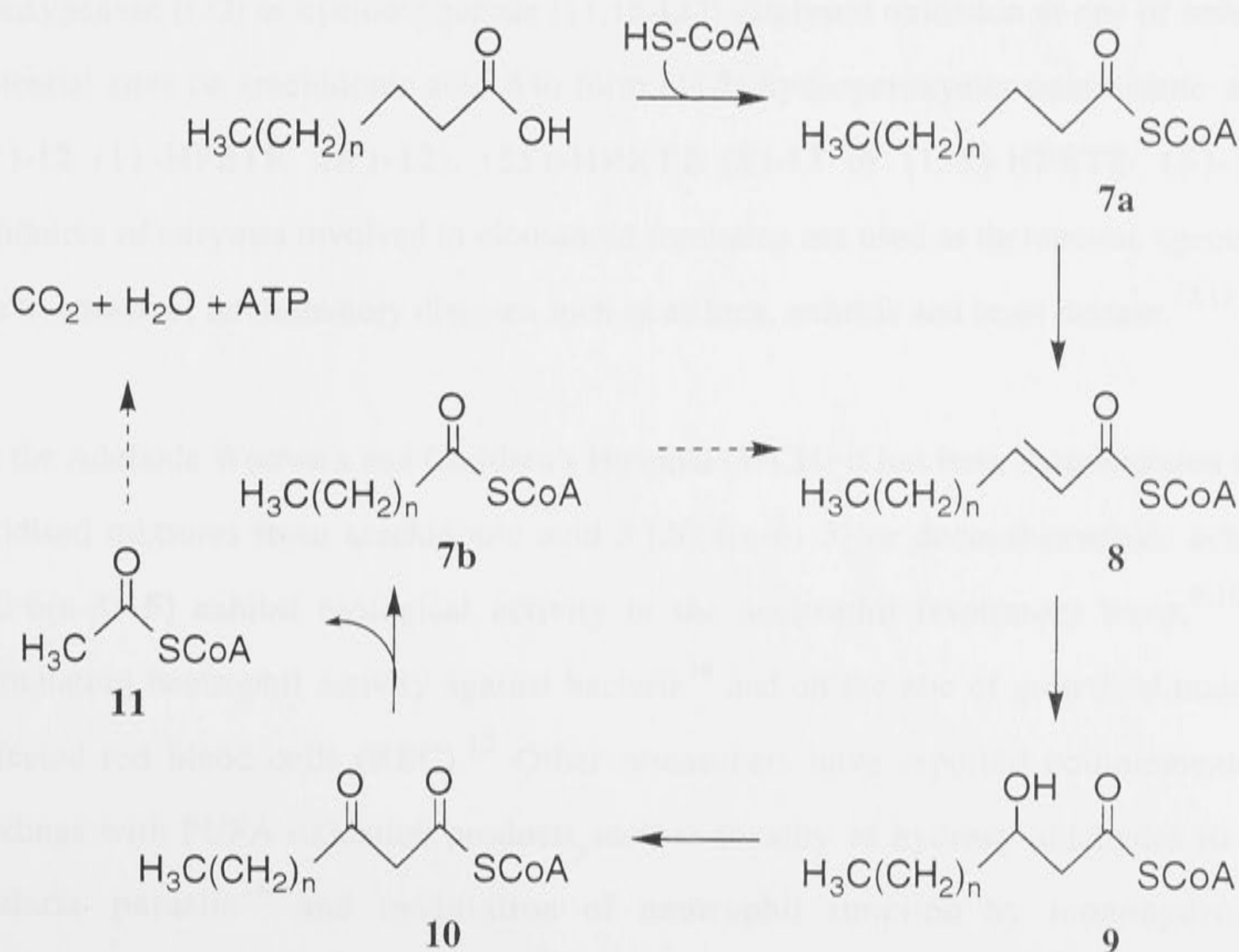
18:3(n-3)

The presence of carbon-carbon double bonds markedly alters the physical properties of unsaturated fatty acids and unsaturated esters in comparison to the saturated forms. For example, the saturated compound stearic acid [18:0] has a melting point of 70 °C, whereas the monounsaturated compound oleic acid [18:1(n-9)] has a melting point of 13 °C.¹ As cell membranes contain high concentrations of esterified fatty acids, the physical properties of these membranes are dependent on the relative amounts of saturated and unsaturated esters present as triglycerides **1**. A diet high in saturated fat has been linked to

elevated plasma cholesterol levels and fatty deposits in the artery wall, which are characteristic of atherosclerosis.⁴ Atherosclerosis results in reduced blood flow, blocked arteries and an increase in the formation of blood clots and is an early stage in the development of coronary heart disease, the leading cause of mortality in Westernised countries.

PUFAs are a source of energy in tissues, providing over twice the yield of energy per gram than that from carbohydrates or protein.^{1,3} Fat reserves are metabolised *via* a fatty acyl-CoA derivative **7a** in the β -oxidation pathway shown in Scheme 1.1. The degradation of fatty acyl-CoA **7a** involves dehydrogenation to enoyl-CoA **8**, hydration to β -hydroxyacyl-CoA **9**, oxidation to β -ketoacyl-CoA **10** and thiolysis to form acetyl-CoA **11** and fatty acyl-CoA **7b** two carbons shorter than the initial fatty acyl-CoA **7a**. The acetyl-CoA **11** produced *via* this pathway is broken down into carbon dioxide and water in the citric acid cycle and the energy released by this process is used to produce adenosine triphosphate (ATP).

Scheme 1.1: The β -oxidation pathway



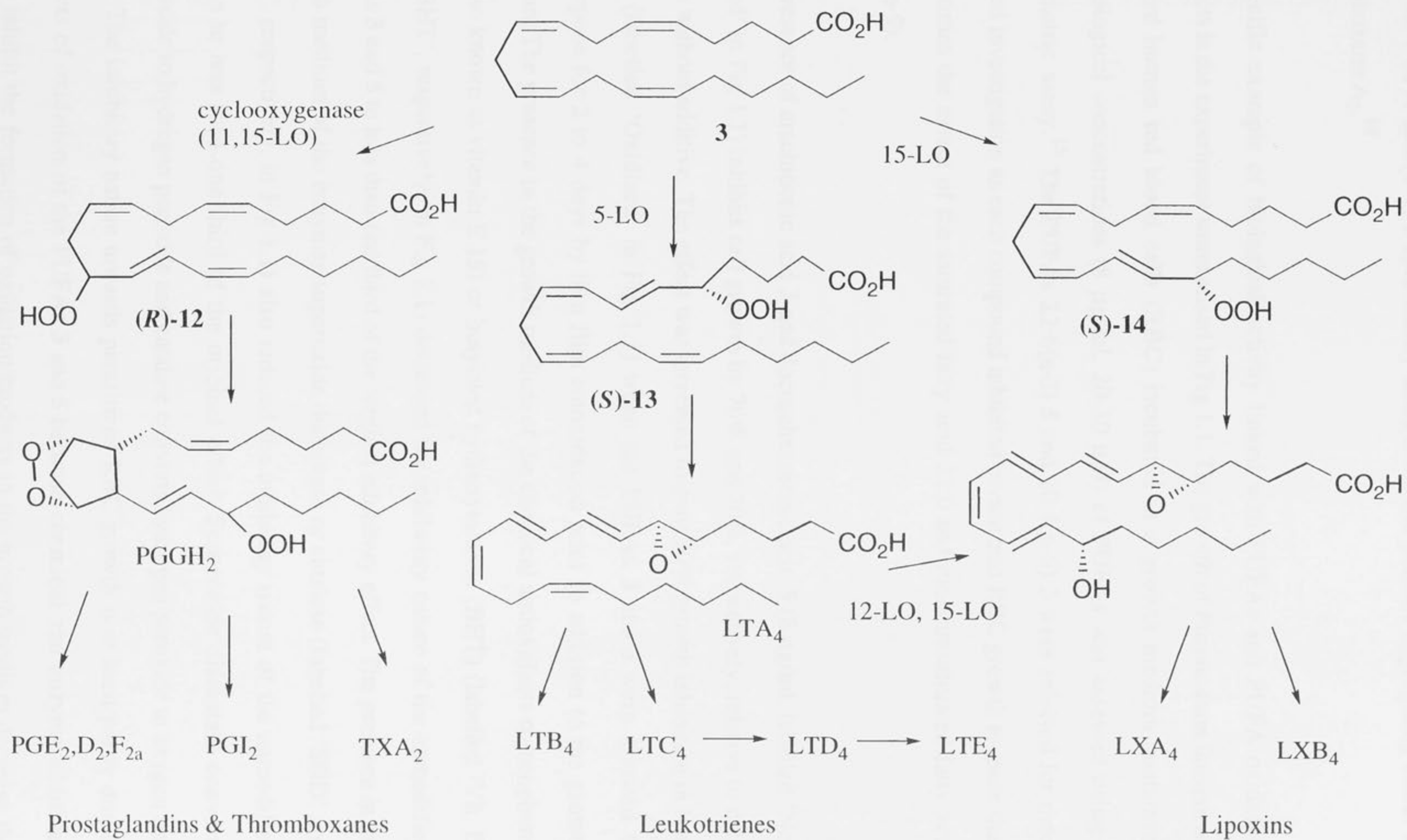
As the fatty acyl-CoA **7b** can be degraded further *via* the β -oxidation pathway, a saturated fatty acid can be entirely broken down to acetyl-CoA **11** in order to produce ATP. PUFAs are also entirely broken down to acetyl-CoA **11** *via* the β -oxidation pathway with the involvement of isomerase enzymes to modify the *cis*-double bonds.

PUFA autoxidation, that is the decomposition of PUFAs exposed to air by oxidation, is relevant to the food industry with regard to the storage of edible oils because PUFA decomposition introduces unfavourable flavours. PUFAs and their oxidised derivatives are also involved in intracellular communication. Examples of effects associated with PUFAs include enzyme activation,⁵ regulation of ion channel function,^{6,7} promotion of gene expression⁸ and activation of neutrophil function.^{9,10} PUFAs induce an increase in cytosolic free calcium (Ca^{2+}),⁶ activate calcium channels in ventricular myocytes⁷ and induce peroxisomal β -oxidation in hepatoma cells.¹¹

Eicosanoids are a class of molecules including prostaglandins, leukotrienes, lipoxins and thromboxanes which have stimulatory and inhibitory roles at the site of origin in organs and tissues (Scheme 1.2).⁴ The first step in the formation of an eicosanoid involves a lipoxygenase (LO) or cyclooxygenase (11,15-LO) catalysed oxidation at one of several potential sites on arachidonic acid **3** to form (11*R*)-hydroperoxyeicosatetraenoic acid (*R*)-**12** (11-HPETE (*R*)-**12**), (5*S*)-HPETE (*S*)-**13** or (15*S*)-HPETE (*S*)-**14**. Inhibitors of enzymes involved in eicosanoid formation are used as therapeutic agents in the treatment of inflammatory diseases such as asthma, arthritis and heart disease.^{12,13}

At the Adelaide Women's and Children's Hospital (WCH) it has been demonstrated that oxidised mixtures from arachidonic acid **3** [20:4(n-6) **3**] or docosahexaenoic acid **5** [22:6(n-3) **5**] exhibit biological activity in the neutrophil respiratory burst,^{9,10} in stimulating neutrophil activity against bacteria¹⁴ and on the rate of growth of malaria infected red blood cells (RBC).¹⁵ Other researchers have reported complementary findings with PUFA oxidation products, such as toxicity of hydroxy-aldehydes to the malaria parasite¹⁶ and modulation of neutrophil function by monohydroxy-

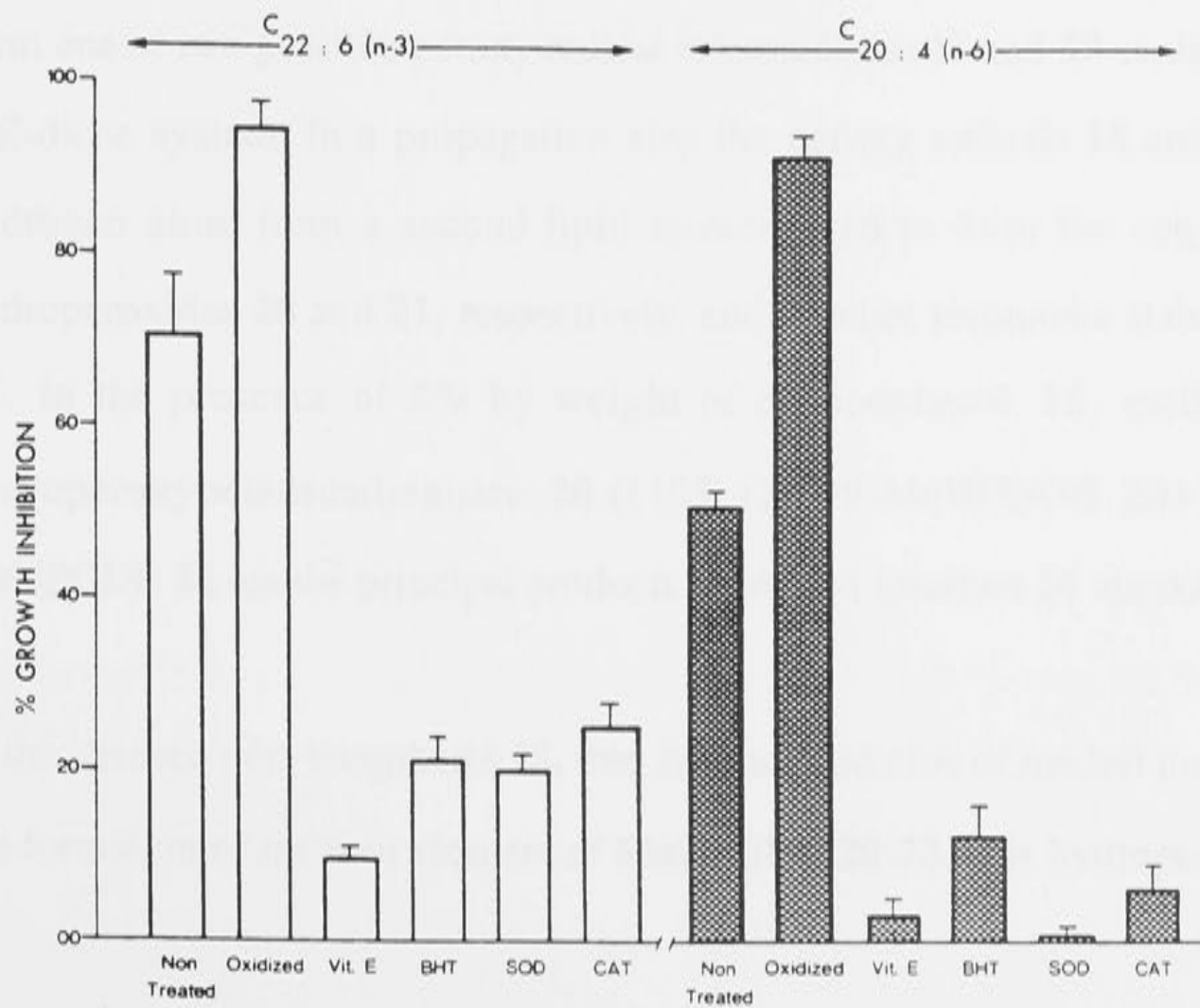
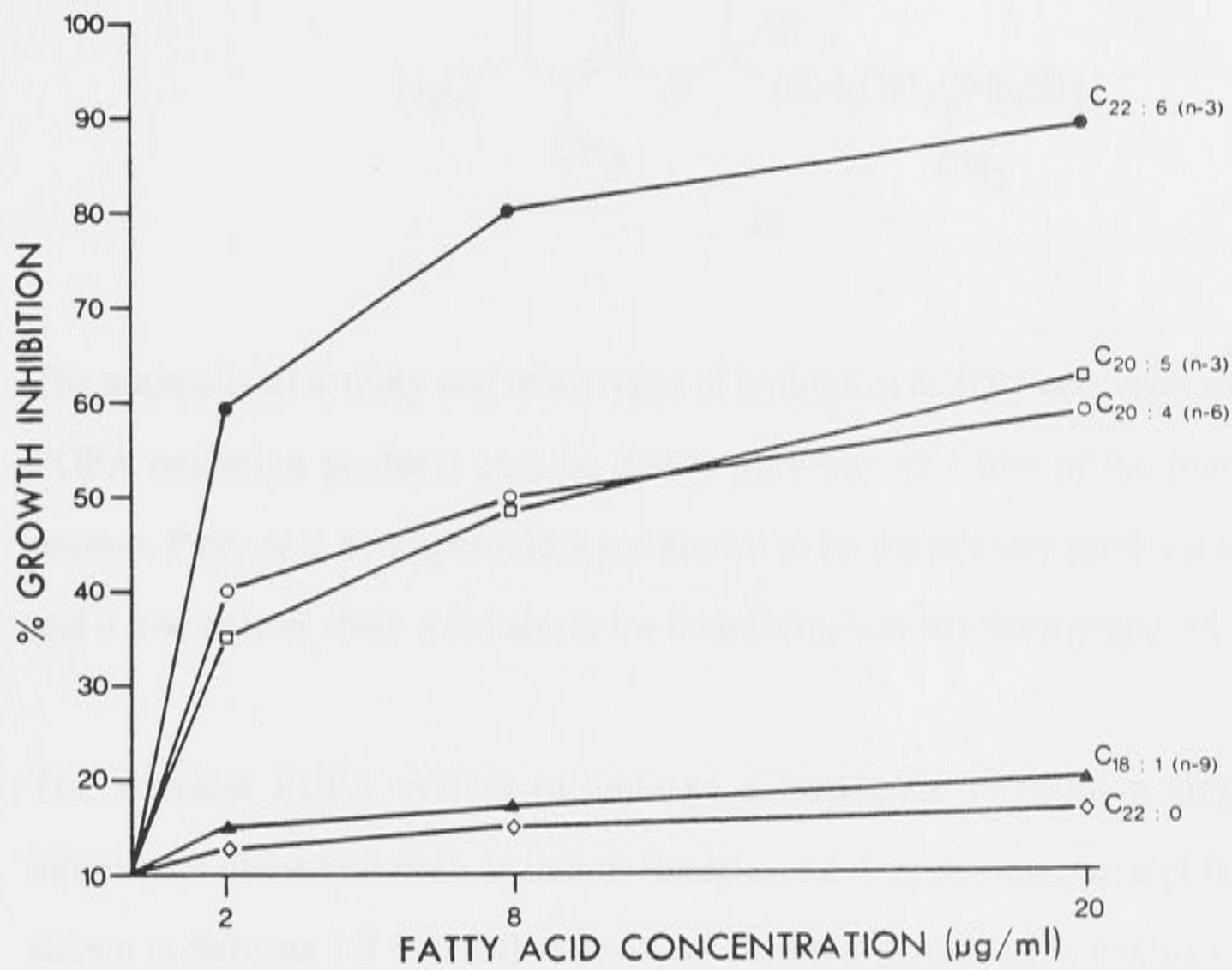
Scheme 1.2: Eicosanoid formation from arachidonic acid **3**

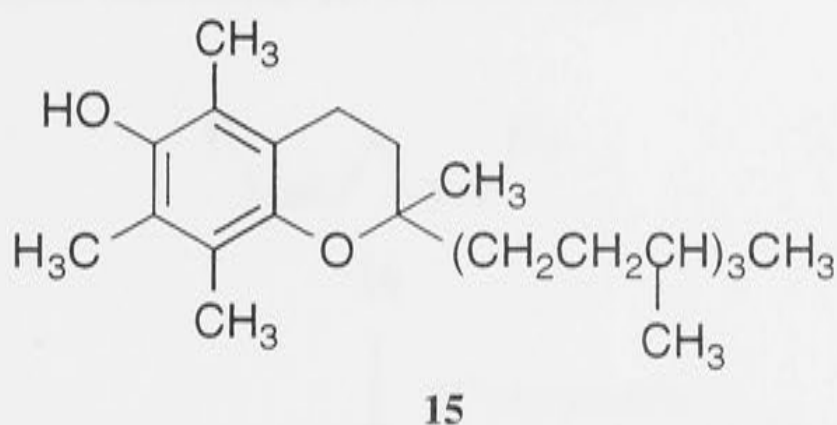


eicosatetraenoic acid isomers.¹⁷ Hydroxy-fatty acids and hydroperoxy-fatty acids derived from the PUFA 22:6(n-3) **5** specifically antagonize the platelet aggregating effect of thromboxane A₂.¹⁸

A specific example of biological activity linked with PUFAs and PUFA oxidation products is the experiment summarised in Fig 1.1. The growth of *Plasmodium falciparum* infected human red blood cells (RBC) incubated in a growth medium containing physiological concentrations (8 µg/ml, 20-30 µM) of PUFAs was assessed using a radiometric assay.¹⁵ The PUFAs 22:6(n-3) **5** and 20:4(n-6) **3** were selected for more detailed investigation as each compound inhibited parasitized RBC growth to more than three times the extent of the saturated fatty acid 22:0 and monounsaturated fatty acid 18:1(n-9).

The presence of arachidonic acid **3** and docosahexaenoic acid **5** (8 µg/ml, labelled 'Non Treated' in Fig 1.1) inhibits cell growth by 70% and 50%, respectively, relative to cells grown without additive. The effect was increased to over 90% growth inhibition in both cases (labelled 'Oxidised' in Fig 1.1) when the PUFAs **3** and **5** were allowed to decompose for 2 to 4 days by thin film autoxidation prior to addition to the growth medium. The presence in the growth medium of the chemical antioxidants α -tocopherol **15** (also known as vitamin E **15**) or butylated hydroxytoluene (BHT) (labelled 'Vit. E' and 'BHT', respectively, in Fig 1.1) decreased the inhibitory nature of the unmodified PUFAs **3** and **5** to less than one third of the original inhibitory effect. The presence in the growth medium of the enzymes superoxide dismutase or catalase (labelled 'SOD' and 'CAT', respectively, in Fig 1.1) also reduced the inhibitory nature of the unmodified PUFAs to less than one third of the original effect. Superoxide dismutase converts superoxide to hydrogen peroxide and catalase converts hydrogen peroxide to oxygen and water. The inhibitory nature towards parasitized RBC growth is at least partly due to products of oxidation of the PUFAs **3** and **5** because chemical and enzyme additives which inhibit the formation of oxidation products in the growth medium decrease the inhibitory effect of the fatty acids **3** and **5**.

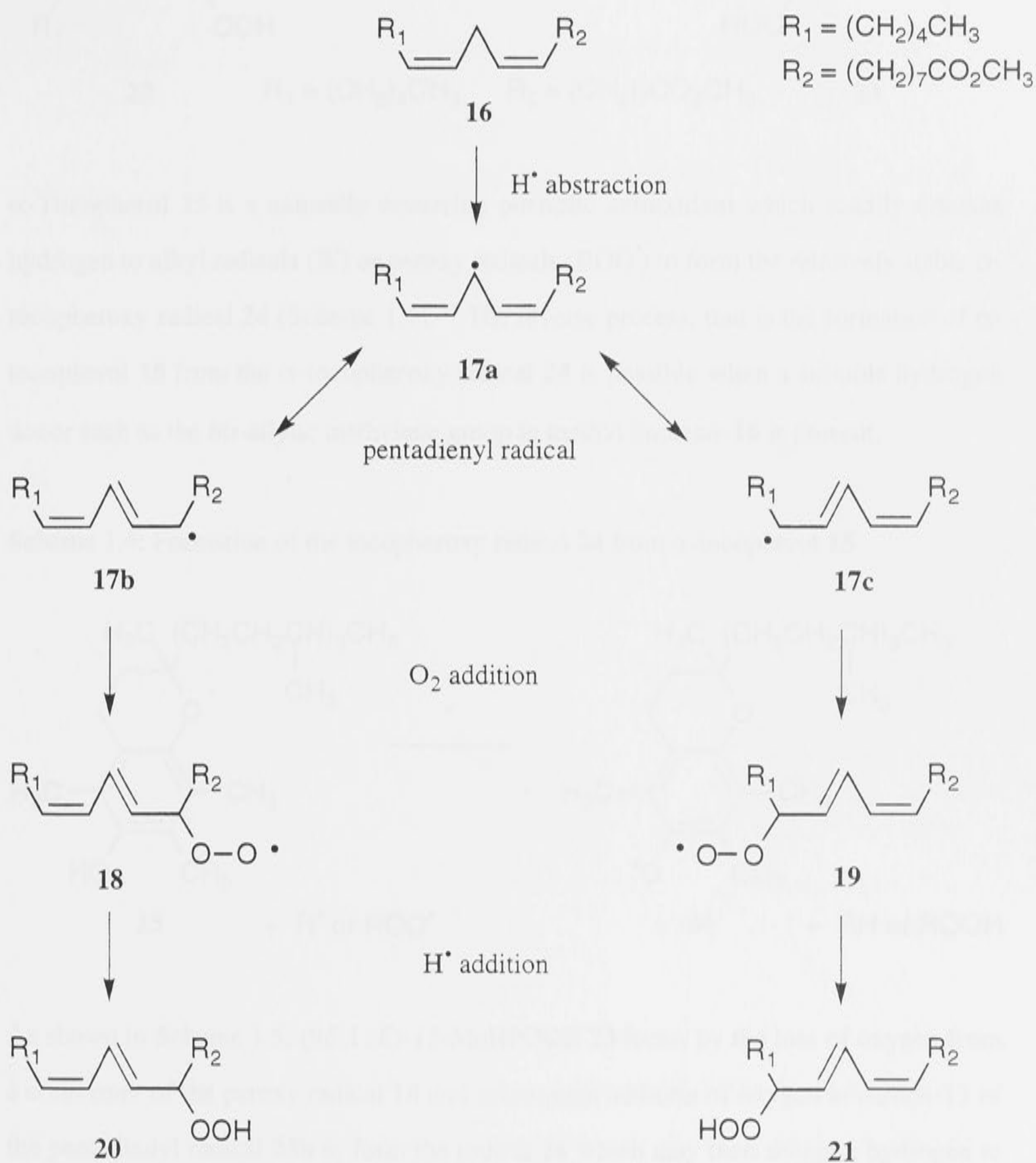
Fig 1.1: Growth inhibition of *Plasmodium falciparum* infected cells by fatty acids



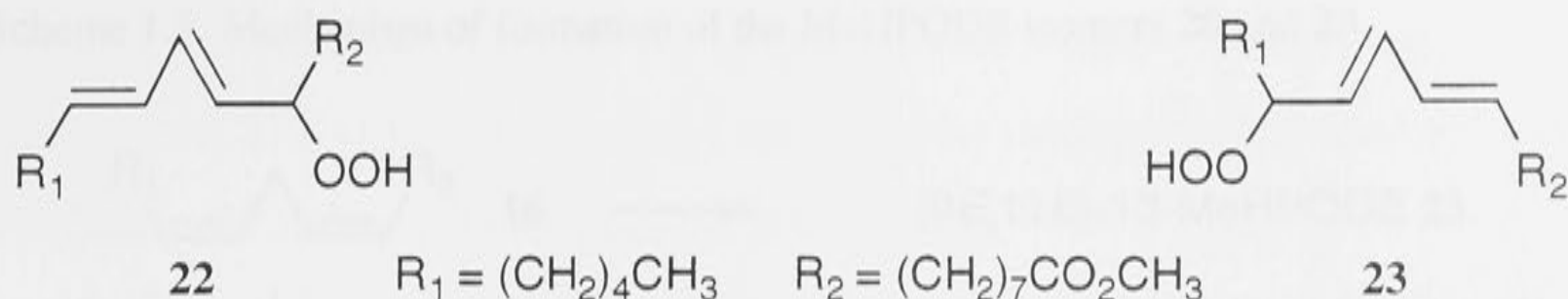
The antimalarial activity and other types of biological activity observed with mixtures of PUFA oxidation products may be due to only one or a few of the many compounds present. Fatty acid hydroperoxides are known to be the primary products of autoxidation and a free-radical chain mechanism for their formation has been proposed.^{19,20,21}

The simplest PUFA system to undergo autoxidation contains a single methylene interrupted diene unit such as that in linoleic acid **4** or the ester methyl linoleate **16**. As shown in Scheme 1.3 for methyl linoleate **16**, lipid peroxidation begins *via* a first chain step in which a hydrogen atom is abstracted from a methylene group of the fatty acid by a sufficiently reactive species such as a hydroxy radical or a peroxy radical. Removal of a hydrogen atom from the *bis*-allylic carbon of methyl linoleate **16** leads to the resonance stabilised pentadienyl radical **17**. Oxygen can add to this alkyl radical intermediate **17** to form one of two possible peroxy radical intermediates **18** and **19** containing a conjugated *E,Z*-diene system. In a propagation step the peroxy radicals **18** and **19** can abstract a hydrogen atom from a second lipid molecule **16** to form the conjugated *E,Z*-diene hydroperoxides **20** and **21**, respectively, and another resonance stabilised alkyl radical **17**. In the presence of 5% by weight of α -tocopherol **15**, methyl (10*E*,12*Z*)-9-hydroperoxyoctadecadienoate **20** ((10*E*,12*Z*)-9-MeHPODE **20**) and (9*Z*,11*E*)-13-MeHPODE **21** are the principal products of methyl linoleate **16** autoxidation.^{22,23}

In the absence of α -tocopherol **15**, thin film autoxidation of methyl linoleate **16** results in the formation of the four isomers of MeHPODE **20-23**. The hydroperoxide isomers **20-**

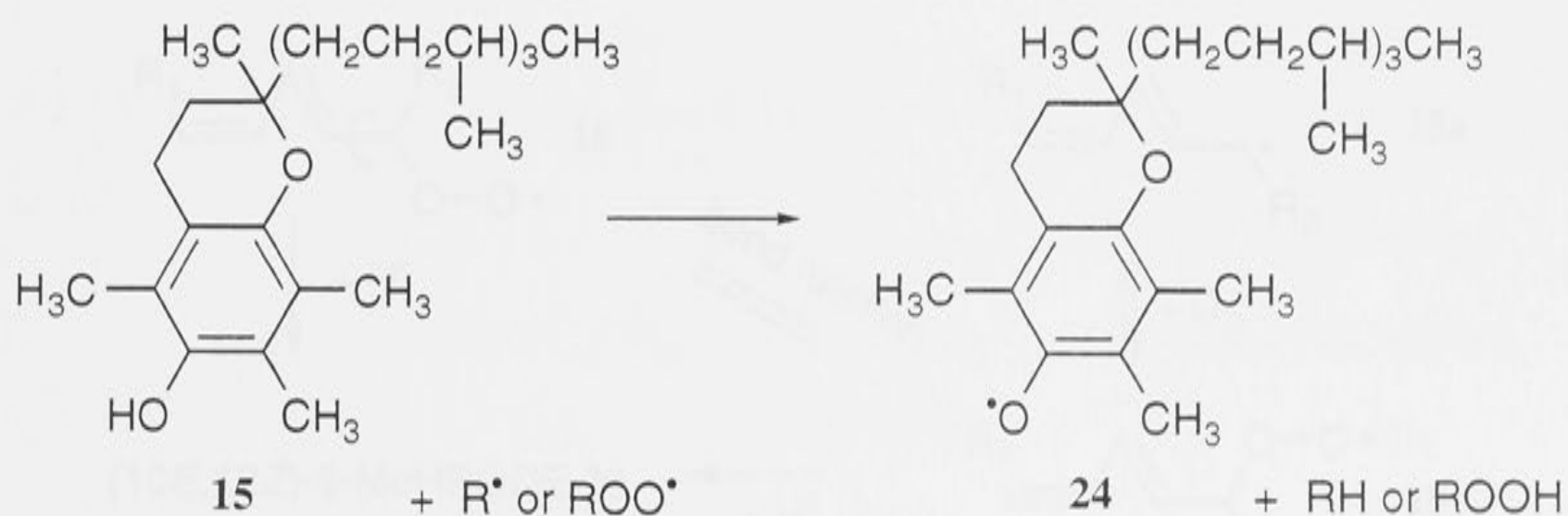
Scheme 1.3: Autoxidation mechanism for methyl linoleate **16**

23 can be reduced to methyl hydroxyoctadecadienoate (MeHODE) isomers and separated by high performance liquid chromatography (HPLC) into regioisomeric pairs of geometric isomers. (10*E*,12*Z*)-9-MeHODE, (10*E*,12*E*)-9-MeHODE, (9*Z*,11*E*)-13-MeHODE and (9*E*,11*E*)-13-MeHODE form in the ratio 21 : 28 : 23 : 28 but under otherwise identical conditions in the presence of 5% α -tocopherol **15** the isomers form in the ratio 49 : 0 : 51 : 0.²²



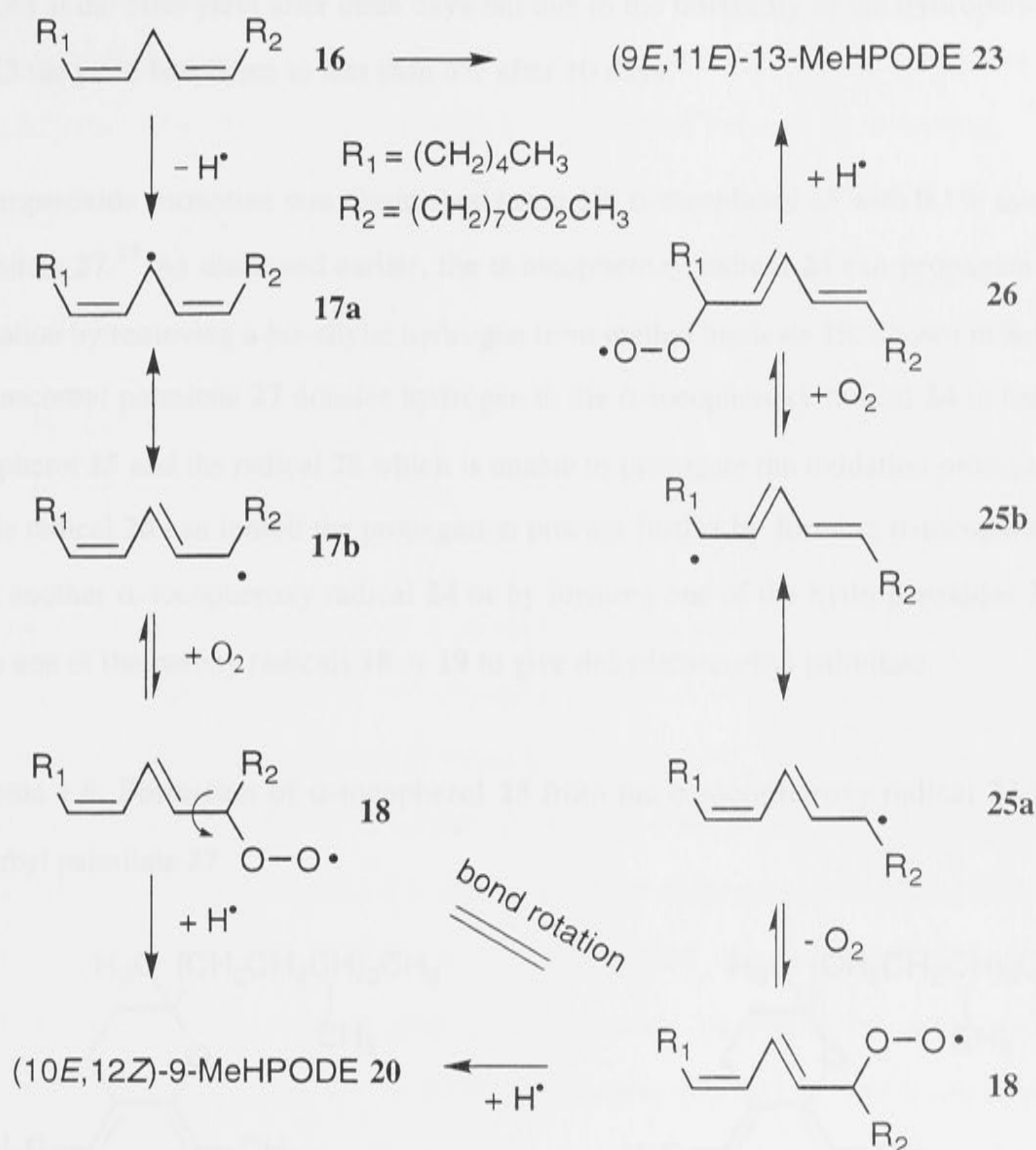
α -Tocopherol **15** is a naturally occurring phenolic antioxidant which readily donates hydrogen to alkyl radicals (R^\bullet) or peroxy radicals (ROO^\bullet) to form the relatively stable α -tocopheroxy radical **24** (Scheme 1.4).²⁴ The reverse process, that is the formation of α -tocopherol **15** from the α -tocopheroxy radical **24** is possible when a suitable hydrogen donor such as the *bis*-allylic methylene group in methyl linoleate **16** is present.

Scheme 1.4: Formation of the tocopheroxy radical **24** from α -tocopherol **15**



As shown in Scheme 1.5, (9*E*,11*E*)-13-MeHPODE **23** forms by the loss of oxygen from a conformer of the peroxy radical **18** and subsequent addition of oxygen at carbon-13 of the pentadienyl radical **25b** to form the radical **26** which may then accept a hydrogen to form the hydroperoxide (9*E*,11*E*)-13-MeHPODE **23**.²⁰ The formation of (10*E*,12*E*)-9-MeHPODE **22** from the radical **19** follows an analogous mechanism.

Using thin film autoxidation with 5% α -tocopherol **15**, the rate of hydrogen transfer from α -tocopherol **15** to the peroxy radicals **18** and **19** is more rapid than the rate of rearrangement to the peroxy radical **26** and the regioisomeric precursor of **22** so the *E,E*-dienes **22** and **23** are not observed in the presence of 5% α -tocopherol **15**. By donating

Scheme 1.5: Mechanism of formation of the MeHPODE isomers **20** and **23**

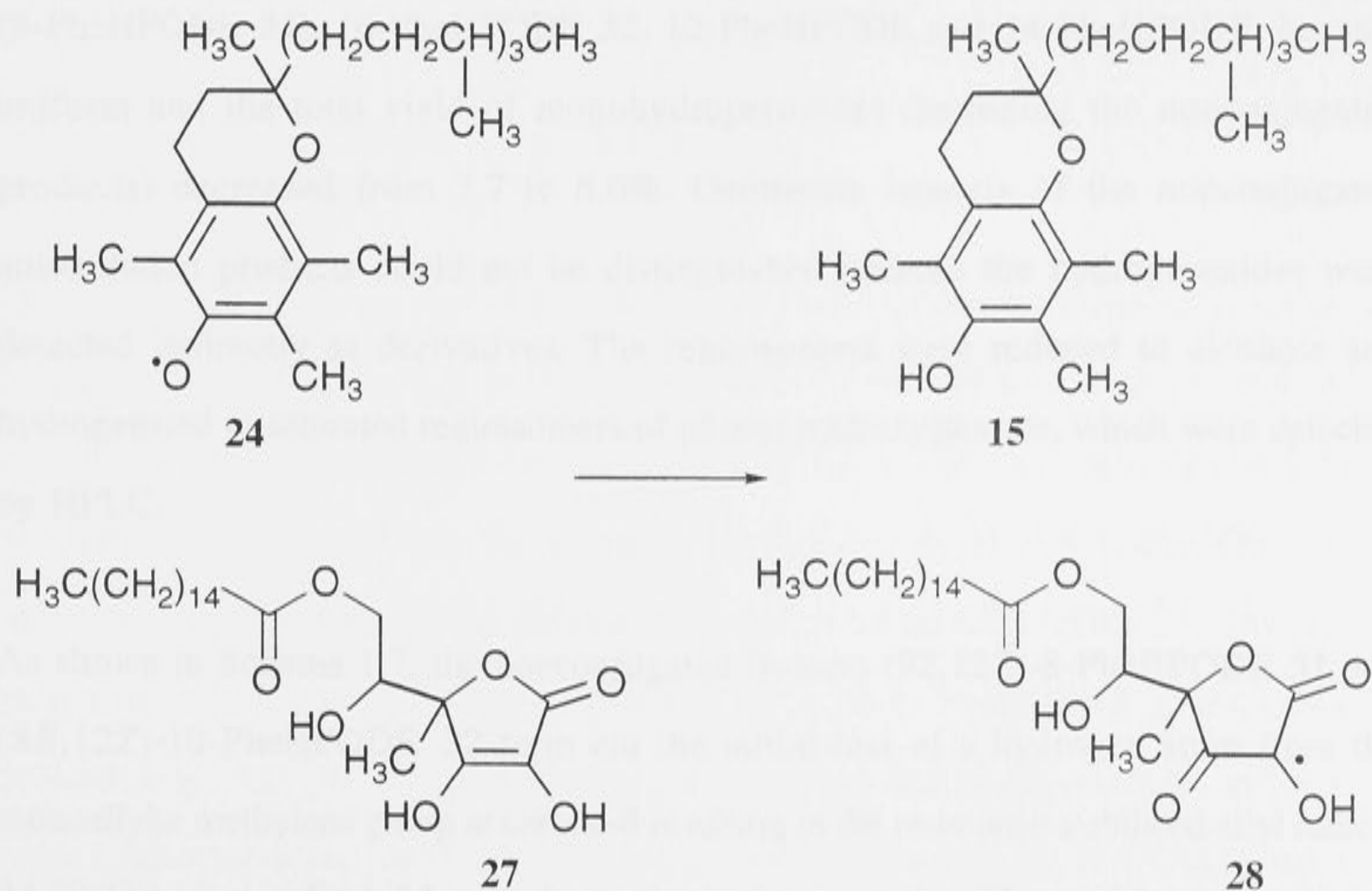
hydrogen to a peroxy radical **18** or **19**, α -tocopherol **15** forms the α -tocopheroxy radical **24**. Whilst α -tocopherol **15** is an antioxidant and slows the oxidation process at lower concentrations, using 5% α -tocopherol **15**, propagation of lipid oxidation can occur via hydrogen abstraction from methyl linoleate **16** by the α -tocopheroxy radical **24**, to form a pentadienyl radical **17** and α -tocopherol **15**.

The effect of α -tocopherol **15** concentrations of 0, 0.01, 0.1 and 1% by weight on the formation of oxidation products from methyl linoleate **16** has been monitored in greater detail. After ten days of thin film oxidation with 1% α -tocopherol **15** the 10% yield of peroxidised material which contained 95% conjugated *E,Z*-diene peroxides exceeded the

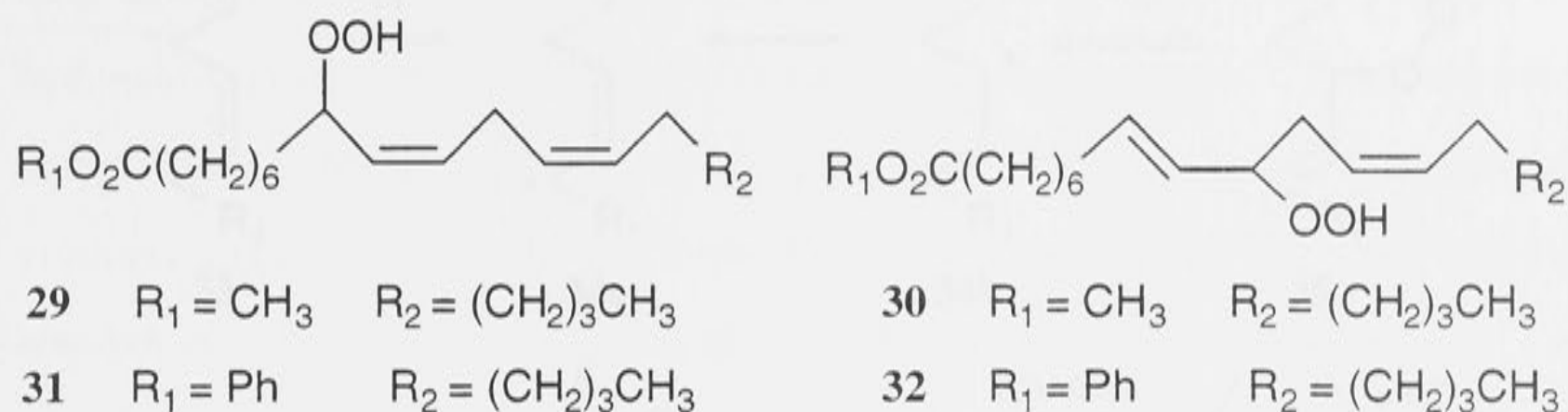
yields for the other samples.²⁵ In the sample without additive, hydroperoxide levels peaked at the 20% yield after three days but due to the instability of the hydroperoxides **20-23** the yield had fallen to less than 5% after 10 days.

Hydroperoxide formation was diminished using 1% α -tocopherol **15** with 0.1% ascorbyl palmitate **27**.²⁵ As discussed earlier, the α -tocopheroxy radical **24** can propagate lipid oxidation by removing a *bis*-allylic hydrogen from methyl linoleate **15**. Shown in Scheme 1.6, ascorbyl palmitate **27** donates hydrogen to the α -tocopheroxy radical **24** to form α -tocopherol **15** and the radical **28** which is unable to propagate the oxidation process. The stable radical **28** can inhibit the propagation process further by forming α -tocopherol **15** with another α -tocopheroxy radical **24** or by forming one of the hydroperoxides **20-23** from one of the peroxy radicals **18** or **19** to give dehydroascorbyl palmitate.

Scheme 1.6: Formation of α -tocopherol **15** from the α -tocopheroxy radical **24** using ascorbyl palmitate **27**



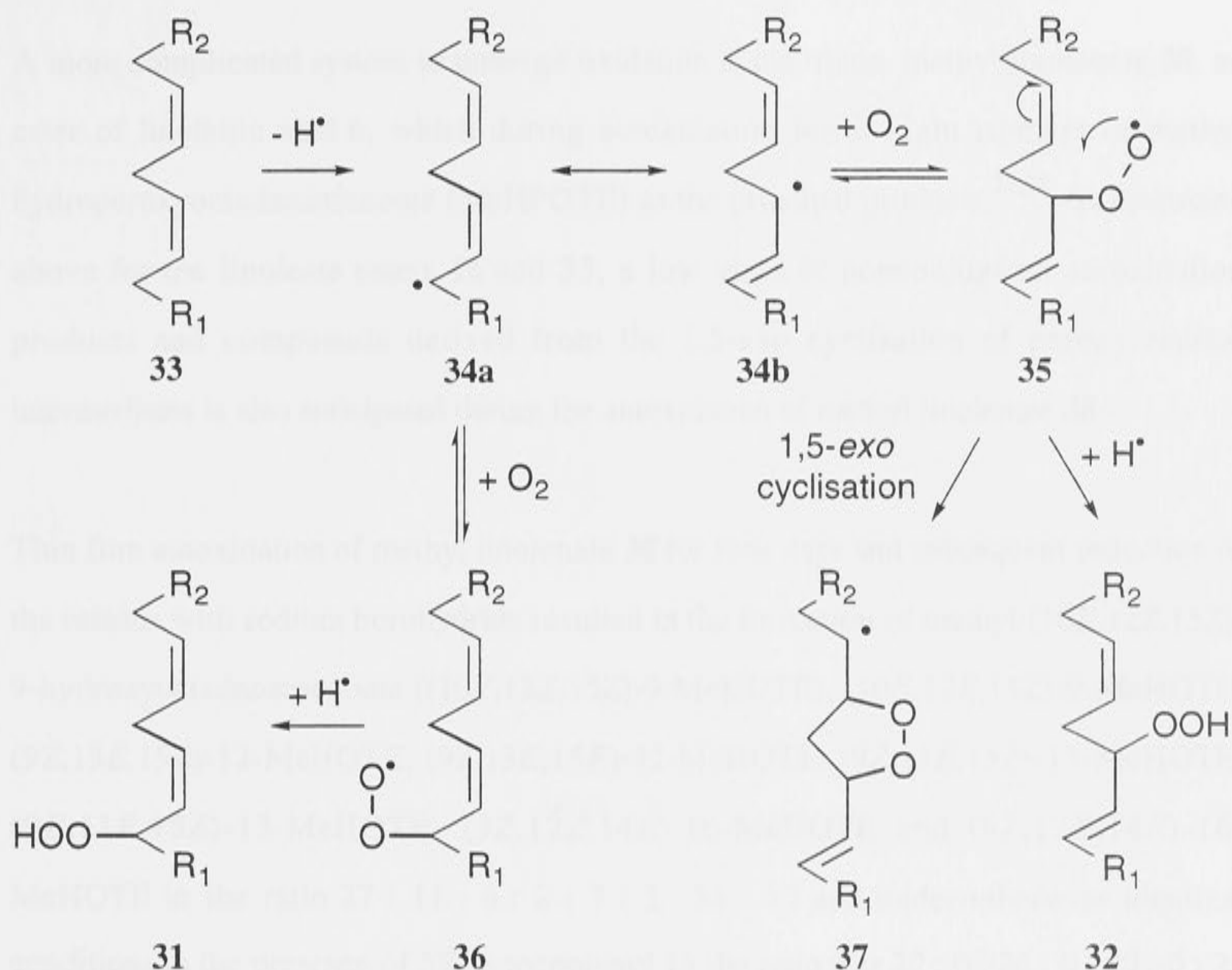
In subsequent experiments it has been shown that the nonconjugated isomers (9Z,12Z)-8-MeHPODE **29** and (9Z,12Z)-14-MeHPODE are also formed by autoxidation of methyl linoleate **16**.²⁶ It is probable that four regioisomeric hydroperoxides form but the isomers (8E,12Z)-10-MeHPODE **30** and (9Z,13E)-12-MeHPODE have not been isolated.²⁷



During the 3 day autoxidation of a second linoleate ester, phenyl linoleate **33**, by increasing the concentration of α -tocopherol **15** from 0 to 5% by weight the yield of conjugated diene hydroperoxides decreased from 7.4 to 5.8%.²⁷ In the same experiment, by increasing the α -tocopherol **15** concentration from 0 to 5% the yield of small amounts of the nonconjugated autoxidation products, phenyl 8-hydroperoxyoctadecadienoate **31** (8-PheHPODE **31**), 10-PheHPODE **32**, 12-PheHPODE and 14-PheHPODE, became uniform and the total yield of monohydroperoxides (including the nonconjugated products) decreased from 7.7 to 6.0%. Geometric isomers of the nonconjugated autoxidation products could not be distinguished because the hydroperoxides were detected indirectly as derivatives. The regioisomers were reduced to alcohols and hydrogenated to saturated regioisomers of phenyl hydroxystearate, which were detected by HPLC.

As shown in Scheme 1.7, the nonconjugated isomers (9Z,12Z)-8-PheHPODE **31** and (8E,12Z)-10-PheHPODE **32** form *via* the initial loss of a hydrogen atom from the monoallylic methylene group at carbon-8 resulting in the resonance stabilised allyl radical **34**.²⁰ The allyl radical **34** may form the hydroperoxides **31** and **32** by successive additions of oxygen and hydrogen. The formation of (9Z,13E)-12-PheHPODE and (9Z,12Z)-14-PheHPODE follows an analogous mechanism.

Scheme 1.7: Mechanism of formation of (9Z,12Z)-8-PheHPODE **31** and (8E,12Z)-10-PheHPODE **32**



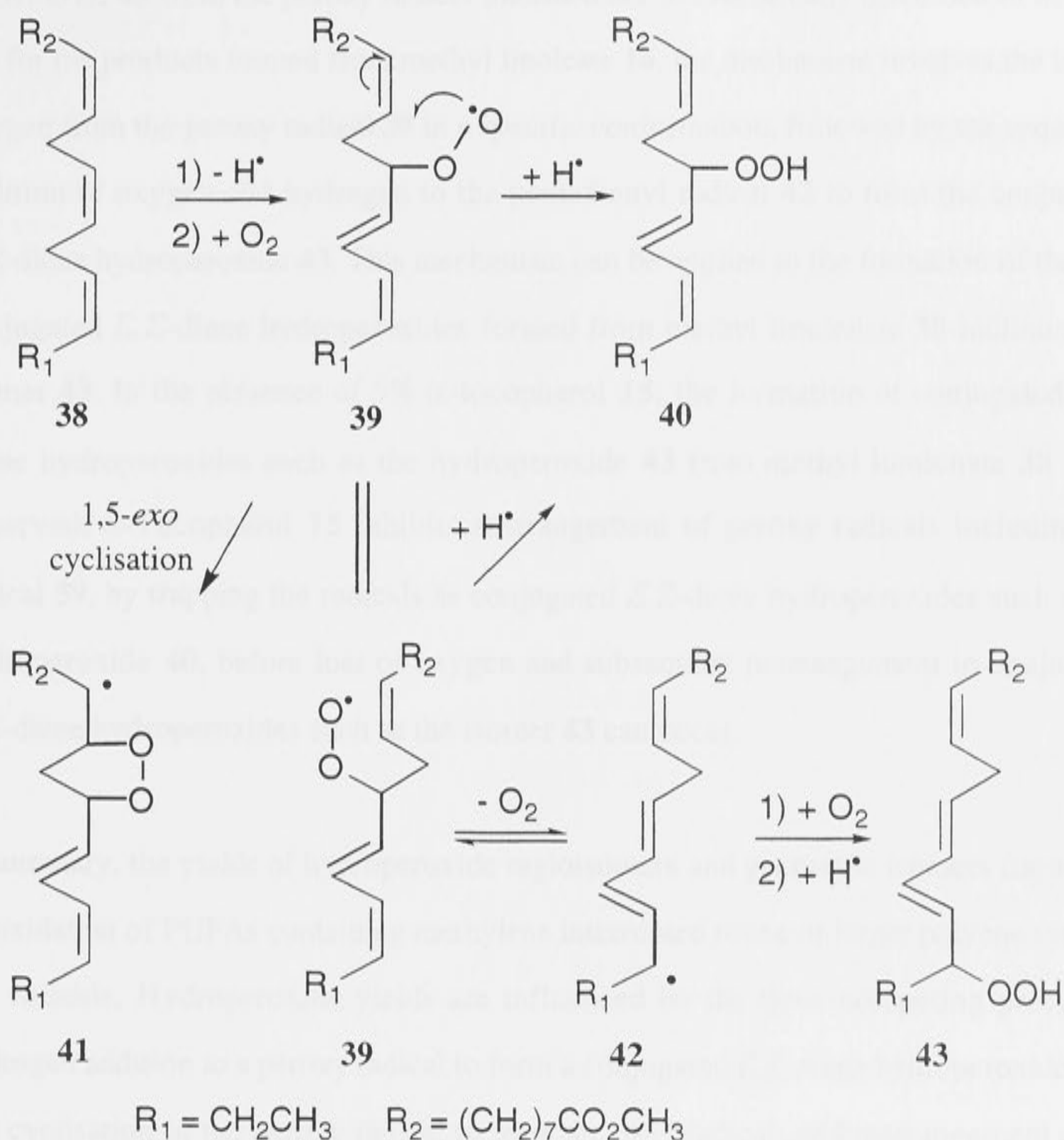
The decomposition of the peroxy radical **35** by 1,5-*exo* cyclisation to the alkyl radical **37** is also shown in Scheme 1.7. In the absence of α -tocopherol **15** the peroxy radical **35** and the regioisomeric precursor of (9Z,13E)-12-PheHPODE may rearrange by 1,5-*exo* cyclisation and consequently the yields of 10-PheHPODE **32** and 12-PheHPODE are about one third of the yields of 8-PheHPODE **31** and 14-PheHPODE.^{23,27} The peroxy radical **36** and the corresponding precursor of (9Z,12Z)-14-PheHPODE do not contain a favourable geometry of peroxy radical and alkene group and consequently do not undergo rapid cyclisation reactions. The yields of 8-PheHPODE and 14-PheHPODE decrease by over 60% in the presence of 5% α -tocopherol **15** and the yields of conjugated diene hydroperoxides decrease by 20% indicating that under these conditions the formation of

all peroxy radicals is diminished because the hydrogen donating nature of the antioxidant **15** inhibits the formation of allyl and pentadienyl radicals.

A more complicated system to undergo oxidation is the triene, methyl linolenate **38**, an ester of linolenic acid **6**, which during autoxidation forms eight isomers of methyl hydroperoxyoctadecatrienoate (MeHPOTE) as the principal products.^{22,28} As discussed above for the linoleate esters **16** and **33**, a low yield of nonconjugated autoxidation products and compounds derived from the 1,5-*exo* cyclisation of peroxy radical intermediates is also anticipated during the autoxidation of methyl linolenate **38**.

Thin film autoxidation of methyl linolenate **38** for four days and subsequent reduction of the residue with sodium borohydride resulted in the formation of methyl (10*E*,12*Z*,15*Z*)-9-hydroxyoctadecatrienoate ((10*E*,12*Z*,15*Z*)-9-MeHOTE), (10*E*,12*E*,15*Z*)-9-MeHOTE, (9*Z*,13*E*,15*Z*)-12-MeHOTE, (9*Z*,13*E*,15*E*)-12-MeHOTE, (9*Z*,11*E*,15*Z*)-13-MeHOTE, (9*E*,11*E*,15*Z*)-13-MeHOTE, (9*Z*,12*Z*,14*E*)-16-MeHOTE and (9*Z*,12*E*,14*E*)-16-MeHOTE in the ratio 27 : 11 : 6 : 2 : 7 : 3 : 31 : 13 and under otherwise identical conditions in the presence of 5% α -tocopherol **15** the ratio was 22 : 0 : 24 : 0 : 29 : 0 : 25 : 0.²¹

In Scheme 1.8, three competing pathways for reaction of the peroxy radical **39** are shown. An analogous mechanism can be used to describe the competing mechanisms to form the hydroperoxide isomers (9*Z*,11*E*,15*Z*)-13-MeHPOTE and (10*E*,12*E*,15*Z*)-9-MeHPOTE. All peroxy radicals, including the isomer **39**, form *via* the loss of hydrogen from a *bis*-allylic methylene group of methyl linolenate **38** to form a resonance stabilised pentadienyl radical followed by addition of oxygen to the pentadienyl radical. The simplest route of product formation involves hydrogen addition to the peroxy radical to form a conjugated *E,Z*-diene hydroperoxide as shown in Scheme 1.8 for the formation of (9*Z*,13*E*,15*Z*)-12-MeHPOTE **40** from the peroxy radical **39**. All conjugated *E,Z*-diene hydroperoxides form *via* an analogous mechanism to that shown for the isomer **40**.

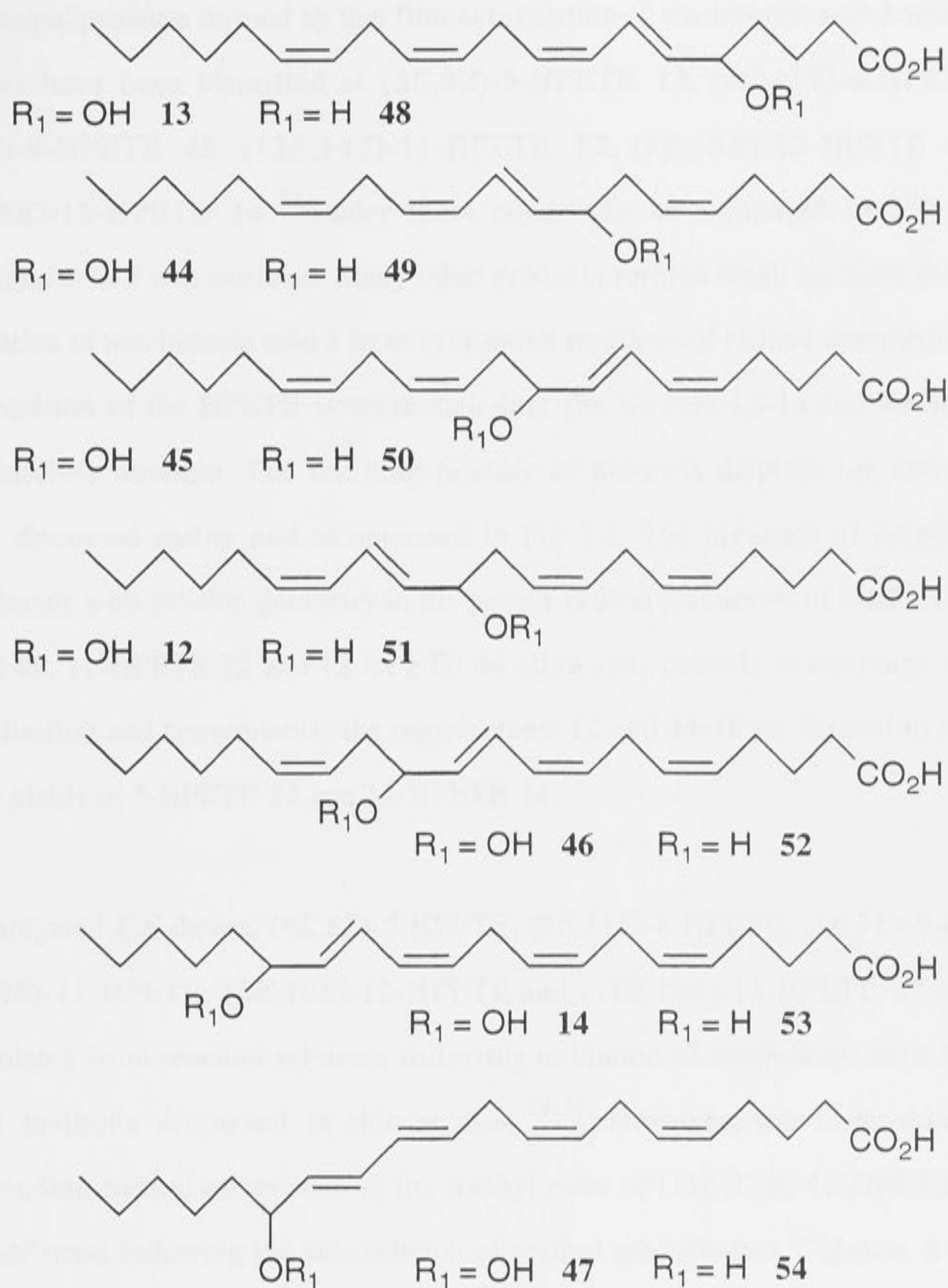
Scheme 1.8: Competing mechanisms of product formation from the peroxy radical **39**

A second route of product formation from the radical **39** involves 1,5-*exo* cyclisation of the peroxy radical **39** to the alkyl radical **41** as shown Scheme 1.8. In the formation of conjugated diene hydroperoxides by autoxidation of methyl linolenate **38**, 1,5-*exo* cyclisation can only occur for the peroxy radical **39** and the analogous precursor of (9*Z*,11*E*,15*Z*)-13-MeHPOTE. In the presence of 5% α -tocopherol **15**, the four conjugated *E,Z*-diene hydroperoxides including the isomer **40** are formed in almost equal amounts indicating that decomposition of the peroxy radical **39** and the radical precursor of (9*Z*,11*E*,15*Z*)-13-MeHPOTE by 1,5-*exo* cyclisation does not occur.²⁰

The third route shown in Scheme 1.8 involves the formation of (9*Z*,12*E*,14*E*)-16-MeHPOTE **43** from the peroxy radical intermediate **39**. As already discussed in Scheme 1.5 for the products formed from methyl linoleate **16**, the mechanism involves the loss of oxygen from the peroxy radical **39** in a specific conformation, followed by the sequential addition of oxygen and hydrogen to the pentadienyl radical **42** to form the conjugated *E,E*-diene hydroperoxide **43**. This mechanism can be applied to the formation of the four conjugated *E,E*-diene hydroperoxides formed from methyl linolenate **38** including the isomer **43**. In the presence of 5% α -tocopherol **15**, the formation of conjugated *E,E*-diene hydroperoxides such as the hydroperoxide **43** from methyl linolenate **38** is not observed. α -Tocopherol **15** inhibits rearrangement of peroxy radicals including the radical **39**, by trapping the radicals as conjugated *E,Z*-diene hydroperoxides such as the hydroperoxide **40**, before loss of oxygen and subsequent rearrangement to conjugated *E,E*-diene hydroperoxides such as the isomer **43** can occur.

In summary, the yields of hydroperoxide regioisomers and geometric isomers formed by autoxidation of PUFAs containing methylene interrupted triene or larger polyene systems are variable. Hydroperoxide yields are influenced by the three competing processes: hydrogen addition to a peroxy radical to form a conjugated *E,Z*-diene hydroperoxide; 1,5-*exo* cyclisation of the peroxy radical to form an alkyl radical; and rearrangement of the peroxy radical leading to formation of a conjugated *E,E*-diene hydroperoxide. The use of the additives α -tocopherol **15** and ascorbyl palmitate **27** has been demonstrated to alter the yields of hydroperoxide regioisomers and geometric isomers formed during autoxidation by influencing the products formed from the intermediate peroxy radicals.

Several methods have been used to prepare oxidation products from arachidonic acid **3**.²⁹⁻³⁴ By analogy with the results for methyl linoleate **16** and methyl linolenate **38**, pairs of geometric isomers of hydroperoxyeicosatetraenoic acid (HPETE) regioisomers including the isomers **12-14** and **44-46** are anticipated as the principal oxidation products derived from arachidonic acid **3**.



For simplicity in this thesis, the two *cis*-double bonds in the tetraene system which remain unmodified during the formation of each HPETE regioisomer from arachidonic acid **3** are not included in the abbreviated name for the compound so that for example (5*Z*,8*Z*,11*Z*,13*E*)-15-HPETE **14** is written as (11*Z*,13*E*)-15-HPETE **14**, and when only conjugated *E,Z*-dienes are being discussed the name is further abbreviated to 15-HPETE **14**. Accordingly, the regioisomer (5*Z*,8*Z*,11*E*,13*E*)-15-HPETE **47** is abbreviated to (11*E*,13*E*)-15-HPETE **47** and remains distinct in name from the geometric isomer (11*Z*,13*E*)-15-HPETE **14**.

The principal products formed by thin film autoxidation of arachidonic acid **3** with air for two days have been identified as (6*E*,8*Z*)-5-HPETE **13**, (9*E*,11*Z*)-8-HPETE **44**, (5*Z*,7*E*)-9-HPETE **45**, (12*E*,14*Z*)-11-HPETE **12**, (8*Z*,10*E*)-12-HPETE **46** and (11*Z*,13*E*)-15-HPETE **14**.²⁹ Under these conditions an estimated 15-25% of the arachidonic acid **3** was oxidised. Many other products form in small amounts during the autoxidation of arachidonic acid **3** from cyclisation reactions of radical intermediates and decomposition of the HPETE isomers including the isomers **12-14** and **44-46** which are themselves unstable. The resulting mixture of products displays the antimalarial activity discussed earlier and summarised in Fig 1.1. The presence of neighbouring double bonds with suitable geometry in the peroxy radical precursors of 8-HPETE **44**, 9-HPETE **45**, 11-HPETE **12** and 12-HPETE **46** allows the radicals to rearrange *via* 1,5-*exo* cyclisation and consequently the regioisomers **12** and **44-46** are formed in less than half the yields of 5-HPETE **13** and 15-HPETE **14**.

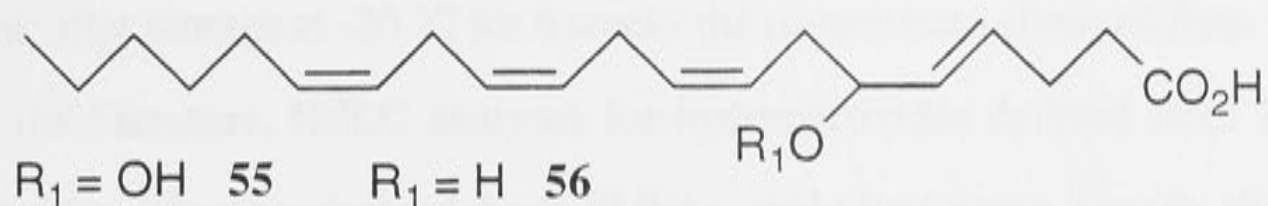
The conjugated *E,E*-dienes, (6*E*,8*E*)-5-HPETE, (9*E*,11*E*)-8-HPETE, (5*E*,7*E*)-9-HPETE, (12*E*,14*E*)-11-HPETE, (8*E*,10*E*)-12-HPETE and (11*E*,13*E*)-15-HPETE **47** have not been isolated from reaction mixtures following oxidation of arachidonic acid **3** *via* the various methods discussed in this section.²⁹⁻³⁴ However, the formation of the corresponding methyl esters such as the methyl ester of (11*E*,13*E*)-15-HPETE **47** has been confirmed following the autoxidation of methyl arachidonate.³⁰ Hence, it is likely that a small proportion of the hydroperoxides formed from arachidonic acid **3** autoxidation are conjugated *E,E*-dienes such as the isomer **47**. Subsequent experiments have indicated that the rate of 1,5-*exo* cyclisation of the peroxy radical precursors of the HPETE isomers **12** and **44-46** is more rapid than the rate of rearrangement to conjugated *E,E*-diene hydroperoxides such as (11*E*,13*E*)-15-HPETE **47**.²⁰

Thin film autoxidation of arachidonic acid **3** in the presence of 5% α -tocopherol **15** resulted in the production of the six conjugated *E,Z*-diene hydroperoxide isomers **12-14** and **44-46** in equal proportions, in 15-20% overall yield.^{30,31} α -Tocopherol **15** acts as a hydrogen donor to the peroxy radical precursors of the conjugated *E,Z*-diene

hydroperoxides **12-14** and **44-46** before the peroxy radical intermediates undergo 1,5-*exo* cyclisation.

Following autoxidation of arachidonic acid **3** (0.24 M) in benzene the conjugated *E,Z*-diene hydroperoxides, 5-HPETE **13**, 8-HPETE **44**, 9-HPETE **45**, 11-HPETE **12**, 12-HPETE **46** and 15-HPETE **14** were isolated in the ratio 27 : 7 : 9 : 11 : 6 : 40.¹⁹ As already discussed for the products formed by thin film autoxidation of arachidonic acid **3**, the peroxy radical precursors of 8-HPETE **44**, 9-HPETE **45**, 11-HPETE **12** and 12-HPETE **46** rearrange *via* 1,5-*exo* cyclisation so the regioisomers **12** and **44-46** are formed in lower yield than 5-HPETE **13** and 15-HPETE **14**. Using arachidonic acid **3** oxidation in solution the radical cyclisation of a peroxy radical has been demonstrated to occur over five times more rapidly than the loss of oxygen and consequently the formation of conjugated *E,E*-diene hydroperoxides such as the isomer **47** is described as not competitive with the cyclisation of peroxy radicals.²⁰

A third method used to generate the conjugated *E,Z*-diene HPETE isomers **12-14** and **44-46** employs photolysis of a solution of arachidonic acid **3** (3 mM) and the photosensitiser methylene blue (180 mg / 500 ml), in methanol at 0 °C, with oxygen bubbling through the solution.³² After 24 to 48 hours under photolysis conditions arachidonic acid **3** and the HPETE isomers **12-14** and **44-46** were separated by HPLC at 0 °C in 38 and 35-40% yields, respectively. The conjugated *E,E*-diene HPETE isomers such as (11*E*,13*E*)-15-HPETE **47** are reported to be absent when arachidonic acid **3** is oxidised using photolysis but are proposed to form following prolonged storage of the conjugated *E,Z*-diene HPETE isomers **12-14** and **44-46** at room temperature. The six HPETE regioisomers **12-14** and **44-46** form in approximately equal amounts. In addition by mass spectrometric analysis the nonconjugated isomers (4*E*,8*Z*)-6-HPETE **55** and (11*Z*,15*E*)-14-HPETE were detected in low yield and coeluted by HPLC with 5-HPETE **13** and 15-HPETE **14**, respectively.



By reduction of photolysis products with sodium borohydride, the hydroxyeicosatetraenoic acid (HETE) isomers **48-53** have been resolved by preparative TLC.³³ The yield of each nonconjugated diene 6-HETE **56** and 14-HETE was half the yield of each conjugated diene 5-HETE **48**, 8-HETE **49**, 9-HETE **50**, 11-HETE **51** and 12-HETE **52** and one third of the yield of the conjugated diene 15-HETE **53**.

A fourth method for the formation of the conjugated *E,Z*-diene HPETE isomers **12-14** and **44-46**, in about 5% yield, required arachidonic acid **3** (10 mM) in methanol, with hydrogen peroxide (0.2 M) and copper chloride (5 mM).³⁴ Following reduction to the HETE isomers **48-53** with sodium borohydride, the mixture of products was separated by HPLC into six fractions containing the isomers 5-HETE **48**, the δ -lactone of 5-HETE, unresolved 8-HETE **49** and 9-HETE **50**, and resolved 11-HETE **51**, 12-HETE **52** and 15-HETE **53** in the ratio 22 : 13 : 22 : 13 : 8 : 22. As already discussed the peroxy radical precursors of 8-HPETE **44**, 9-HPETE **45**, 11-HPETE **12** and 12-HPETE **46** rearrange via 1,5-*exo* cyclisation so the regioisomers **45-46** are formed in lower yield than 5-HPETE **13** and 15-HPETE **14**. The combined yield of 5-HETE **48** and the δ -lactone of 5-HETE exceeds the yield of 15-HETE **53** indicating a preference for oxygen insertion at carbon-5 using this method. The carboxyl group may have a tendency to chelate to the copper catalyst positioning carbon-5 closer to the catalyst than carbon-15 and increasing the incidence of oxidation at carbon-5.

In the literature procedure for the purification of the HPETE isomers **12-14** and **44-46** by HPLC at 0 °C it was noted that considerable hydroperoxide decomposition occurred at room temperature.³² A procedure outlining the isolation of 15-HPETE **14** stated that at room temperature the loss due to decomposition during chromatography is at least 65%.³⁵ Following storage at 42 °C for 40 hours in hexane the ratio of 9-MeHPODE **20** and **22** to 13-MeHPODE **21** and **23** changed from 5 : 95 initially to 41 : 59 and in a second

experiment, after storage at -20°C for 6 weeks the isomer ratio changed from 95 : 5 to 73 : 27.³⁶ In the literature, HPLC analyses for hydroperoxides derived from PUFAs and hydroperoxides of esters derived from PUFAs, including those already discussed for oxidation products of methyl linoleate **16** and methyl linolenate **38**, commonly involve derivatisation of hydroperoxides to the more stable hydroxy-derivatives, by reduction, prior to analysis.^{22,23,37} Reduction of the HPETE isomers **12-14** and **44-46** to the HETE isomers **48-53** with sodium borohydride before characterisation involves a simple procedure and improves the stability of the oxidised fatty acid. Isolation and analysis of the regioisomers and geometrical isomers of HETE **48-53** has been demonstrated using unmodified silica,³² reverse phase³⁸ and chiral phase HPLC.^{37,39}

Differences between the spectroscopic properties of arachidonic acid **3** and each of the HETE isomers including the isomers **48-53** make it possible to identify an isomer. By infra-red (IR) spectroscopy conjugated *E,E*-diene fatty acids have a single absorption band at 990 cm^{-1} whereas conjugated *E,Z*-diene fatty acids have a second absorption band at 950 cm^{-1} .²³ There are no absorption peaks in this region of the IR spectrum for arachidonic acid **3** because there is no conjugated diene system in the molecule.

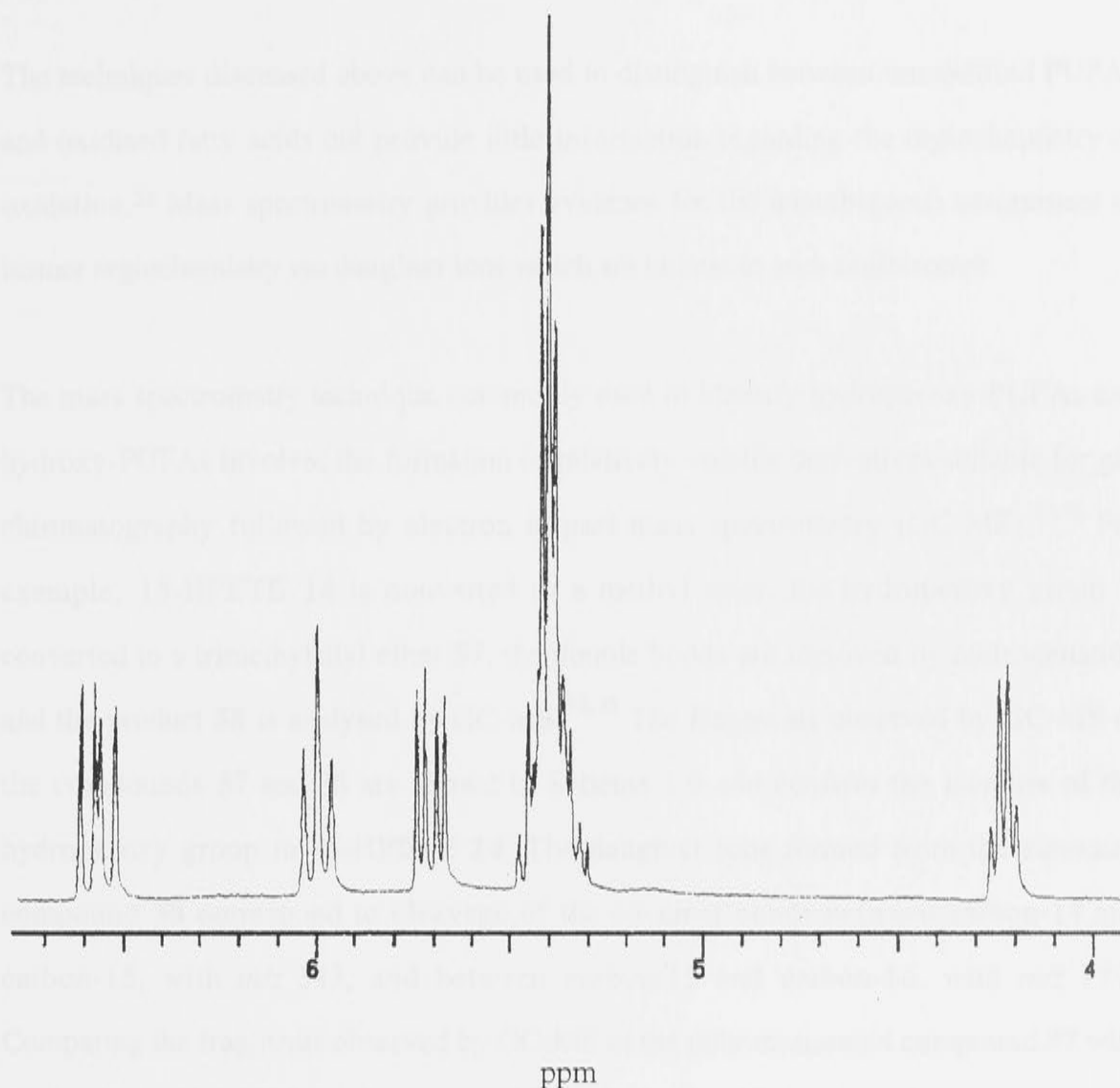
In the ultra-violet (UV) spectra of the six conjugated *E,Z*-diene HETE isomers **48-53**, identical spectra are observed for the pairs 5-HETE **48** and 15-HETE **53**, 8-HETE **49** and 12-HETE **52**, and 9-HETE **50** and 11-HETE **51**, as a result of the symmetry of the polyene system.³¹ The maximum absorption wavelengths (λ_{max}) for the pairs of isomers **48** and **53**, **49** and **52**, and **50** and **51**, are 235, 236 and 237 nm, respectively, and the extinction coefficients (ϵ) are equal at 23000 M^{-1} . Geometrical isomers of a regioisomer can be distinguished by a small difference in λ_{max} and a change in the extinction coefficient. For instance, λ_{max} equals 234 nm for (10*E*,12*Z*)-9-MeHPODE **20** whereas λ_{max} equals 231 nm for (10*E*,12*E*)-9-MeHPODE **22** and the extinction coefficient increases by 10% from 25900 M^{-1} to 28600 M^{-1} .²³ Arachidonic acid **3** does not contain the conjugated diene chromophore so it absorbs UV light relatively poorly. The absorption maxima between 235 and 237 nm are used to detect the HETE isomers **48-53**

following separation by HPLC and to monitor the formation of oxidation products such as the HPETE isomers **12-14** and **44-46** over time.

The proton nuclear magnetic resonance (nmr) spectra of unmodified PUFAs have characteristic signals between 2.0-2.2, 2.7-2.9 and 5.3-5.5 parts per million (ppm) for mono-allylic, *bis*-allylic and olefinic protons, respectively.⁴⁰⁻⁴² Arachidonic acid **3** contains 32 hydrogens and characteristic signals are observed for 4 monoallylic hydrogens, 6 *bis*-allylic hydrogens and 8 olefinic hydrogens at the chemical shift values described above. The remaining proton nmr signals are a triplet at 0.9 ppm for the terminal methyl group, multiplets between 1.6-1.8 ppm and 2.3-2.5 ppm for the methylene groups at carbon-3 and carbon-2, respectively, and a broad multiplet between 1.2-1.4 ppm for the remaining 6 methylene hydrogens. The signal for the carboxylic acid hydrogen is observed with a variable chemical shift value depending on the solvent, the amount of water in the sample and the concentration of the sample.

Characteristic peaks are observed in the proton nmr spectra of the HETE isomers **48-53** compared to arachidonic acid **3**. As shown in Fig 1.2 for 15-HETE **53**, the olefinic hydrogens are observed as four distinct signals in the area ratio 5 : 1 : 1 : 1 between 5.1-6.6 ppm and a signal at 4.2 ppm results from the hydrogen sharing a monoallylic carbon with an alcohol.^{26,42} The proton nmr spectrum of each of the HETE isomers **48-53** is similar making the confident assignment of regiochemistry to an isomer impossible using this technique. The geometry of a double bond can be assigned from the proton nmr spectrum because within an *E*-alkene the vicinal coupling constant $^3J_{HH}$ of the olefinic hydrogens is between 12 and 18 Hz whereas within a *Z*-alkene the constant $^3J_{HH}$ is between 0 and 12 Hz.^{26,42} For instance, $^3J_{HH}$ is 10.7 Hz for the signals resulting from the *Z*-olefinic protons at carbon-9 and carbon-10 in methyl (9*Z*,12*Z*)-8-hydroxyoctadecadienoate ((9*Z*,12*Z*)-8-MeHODE),²⁶ whereas $^3J_{HH}$ is 13.0 Hz for the signals resulting from the *E*-olefinic protons at carbon-9 and carbon-10 in methyl (9*E*,11*Z*)-8-hydroxyeicosatetraenoate (8-MeHETE).⁴²

Fig 1.2: The ^1H nmr spectrum of 15-HETE **53** between 4.0 and 6.7 ppm



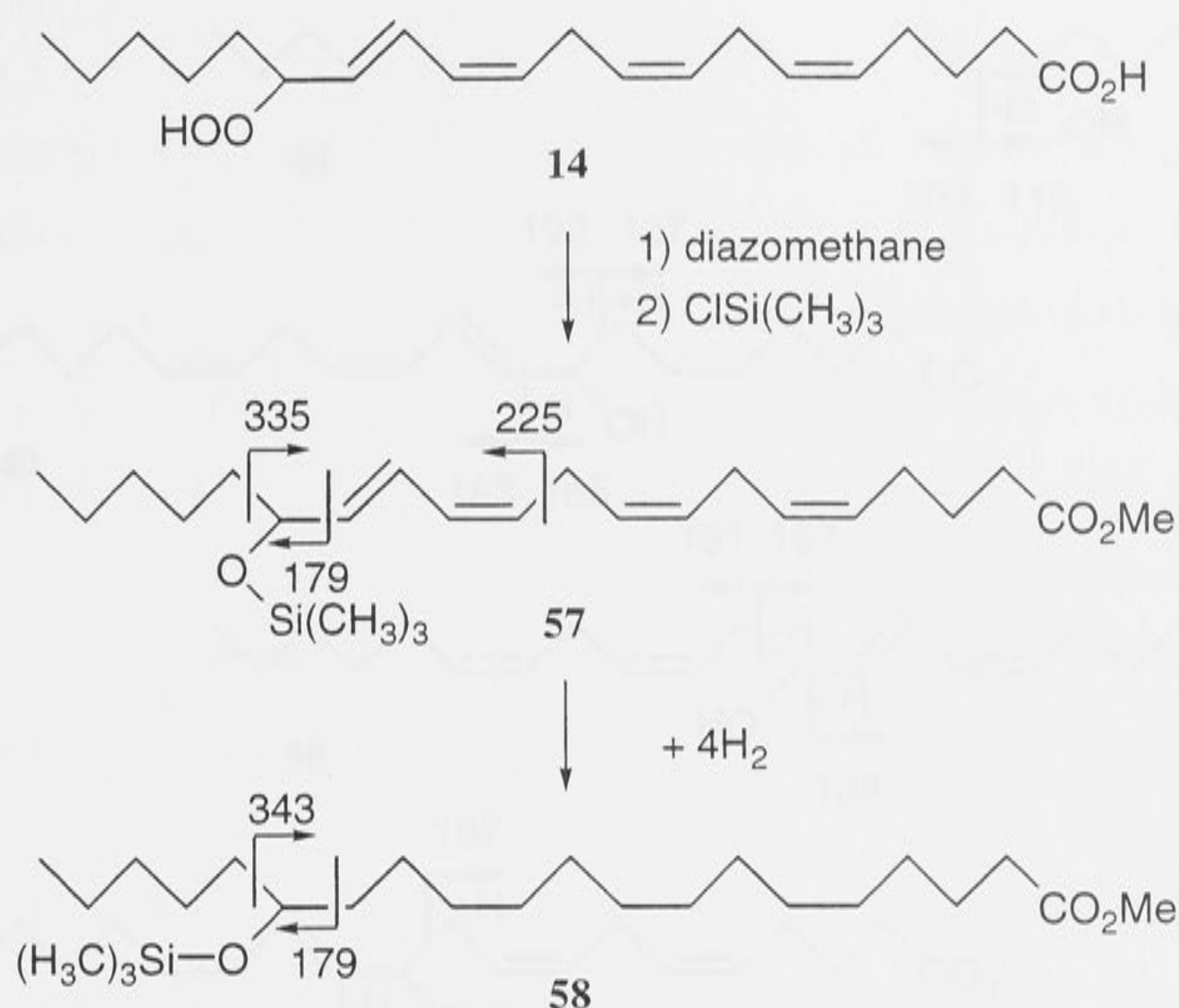
In the ^{13}C nmr spectrum for arachidonic acid **3**, characteristic signals are observed between 127-132 and at 174 ppm for the olefinic and carboxylic acid carbons, respectively. Signals are observed for carbon-2 and carbon-20, at 34 and 14 ppm, respectively, and the remaining carbons are represented by 9 signals between 20-30 ppm.⁴¹ A pair of PUFA geometric isomers can be distinguished based on the chemical shift value observed for the monoallylic carbons because the signals occur between 32-33 or 27-28 ppm when the carbon is adjacent to a *trans*-alkene or a *cis*-alkene, respectively.⁴³ Although the ^{13}C nmr spectra of the HETE isomers **48-53** are yet to be reported, a distinctive feature of the ^{13}C nmr spectra is likely to be a signal between 65-85 ppm for the carbon attached to the alcohol group.⁴⁴ The ^{13}C nmr spectra of PUFAs

cannot be used to confidently distinguish between regioisomers and the same result is expected for the spectra of the HETE isomers **48-53**.

The techniques discussed above can be used to distinguish between unmodified PUFAs and oxidised fatty acids but provide little information regarding the regiochemistry of oxidation.²⁶ Mass spectrometry provides evidence for the unambiguous assignment of isomer regiochemistry *via* daughter ions which are unique to each regioisomer.

The mass spectrometry technique commonly used to identify hydroperoxy-PUFAs and hydroxy-PUFAs involves the formation of relatively volatile derivatives suitable for gas chromatography followed by electron impact mass spectrometry (GC-MS).^{45,46} For example, 15-HPETE **14** is converted to a methyl ester, the hydroperoxy group is converted to a trimethylsilyl ether **57**, the double bonds are removed by hydrogenation and the product **58** is analysed by GC-MS.^{32,35} The fragments observed by GC-MS of the compounds **57** and **58** are shown in Scheme 1.9 and confirm the location of the hydroperoxy group in 15-HPETE **14**. The daughter ions formed from the saturated compound **58** correspond to cleavage of the covalent bonds between carbon-14 and carbon-15, with m/z 343, and between carbon-15 and carbon-16, with m/z 179. Comparing the fragments observed by GC-MS of the polyunsaturated compound **57** with the fragments formed from the saturated lipid derivative **58** confirms that the compound **57** contains four double bonds.

Advances have been made in the period since this project commenced allowing the characterisation of the unmodified HETE **48-53** isomers by several mass spectrometry techniques. Each technique relies on the formation of characteristic daughter ions for each isomer resulting from carbon-carbon bond cleavage adjacent to the hydroxyl group. In 1994, Zhang *et al.*⁴⁷ used negative liquid secondary ion tandem mass spectrometry to characterised unmodified HETE isomers and confirmed the location of the hydroxyl group in 5-HETE **48**, 12-HETE **52** and 15-HETE **53**. In 1995, Wheelan and

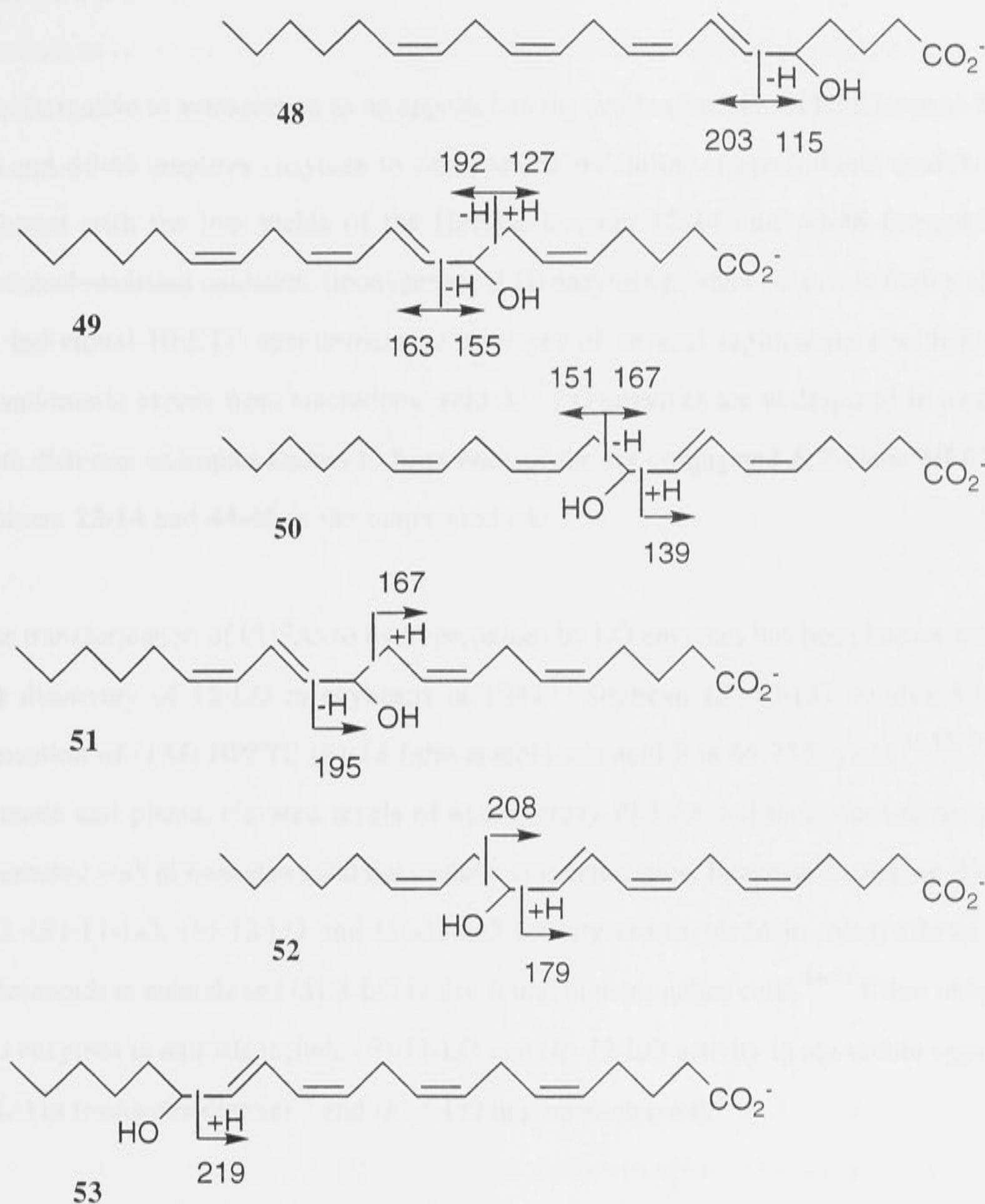
Scheme 1.9: Derivatisation of 15-HPETE **14** and observed MS fragments

Murphy⁴⁸ used negative ion electrospray tandem mass spectrometry to confirm the structure of leukotriene B_4 , a dihydroxylated derivative of arachidonic acid **3**.

In 1993, Wheelan *et al.*^{49,50} published a method to characterise the unmodified HETE regioisomers **48-53** using low-energy negative ion fast atom bombardment tandem mass spectrometry (FAB-MS/MS). The observed fragmentation patterns for the HETE isomers **48-53** by low-energy FAB-MS/MS provide the information to assign regiochemistry and are summarised in Fig 1.3. From one to four characteristic daughter ions clearly identify the location of the hydroxyl group on each regioisomer because each daughter ion forms by cleavage of a covalent carbon-carbon bond on either side of the carbon attached to the hydroxyl group.

To summarise the analytical techniques used to characterise the HPETE isomers **12-14** and **44-46**, derivatisation to the HETE isomers **48-53** is required to assure long term stability of samples and several techniques are required to distinguish between arachidonic

Fig 1.3: MS/MS fragments used to assign regiochemistry to the HETE isomers 48-53



acid **3**, the HETE regioisomers and HETE geometric isomers. UV spectroscopy confirms the presence of a conjugated diene system whilst IR spectroscopy can distinguish between isomers with conjugated *E,Z*-diene or conjugated *E,E*-diene systems. Proton nmr spectroscopy distinguishes the number and ratio of olefinic, *bis*-allylic and monoallylic hydrogens from the remaining hydrogens in a molecule and like ^{13}C nmr spectroscopy can be used to confirm the geometry of the conjugated *E,Z*-diene system. Mass

spectrometry is required to identify HETE regioisomers but fails to distinguish between geometric isomers.

An alternative to autoxidation as an approach to the synthesis of the HPETE isomers **12-14** and **44-46** employs enzymes to catalyse the oxidation of arachidonic acid **3**. In contrast with the low yields of the HPETE isomers **12-14** and **44-46** formed by chemical mediated oxidation, lipoxygenase (LO) enzymes produce relatively high yields of individual HPETE enantiomers or mixtures of several regioisomers with high enantiomeric excess from arachidonic acid **3**.³⁷ LO enzymes are widespread in nature with different examples known to form each of the six conjugated *E,Z*-diene HPETE isomers **12-14** and **44-46** as the major product.

The transformation of PUFAs to hydroperoxides by LO enzymes has been known since the discovery of 15-LO in soybeans in 1947.⁵¹ Soybean (*S*)-15-LO catalyses the formation of (15*S*)-HPETE (*S*)-**14** from arachidonic acid **3** in 65-75% yield.^{35,52,53} In animals and plants, elevated levels of hydroperoxy-PUFAs and their derivatives are associated with disease states and intracellular communication. Enzymes displaying (*S*)-5-LO, (*R*)-11-LO, (*S*)-12-LO and (*S*)-15-LO activity are involved in the synthesis of eicosanoids in animals and (*S*)-8-LO is also found in mammalian cells.⁵⁴⁻⁵⁶ Other unique LO enzymes in animals include (*R*)-11-LO and (*R*)-12-LO activity in sea urchin eggs,⁵⁷ 9-LO in freshwater diatoms⁵⁸ and (*R*)-8-LO in gorgonian coral.⁵⁹

There are many sources of plant LOs,³⁷ such as potato (*S*)-5-LO,^{60,61} wheat (*S*)-8-LO,⁶² rice (*S*)-11-LO,^{63,64} tomato (*S*)-12-LO⁶⁵ and soybean (*S*)-15-LO.³⁵ An isozyme of LO from pea forms almost equal proportions of racemic 5-HPETE **13**, 9-HPETE **45**, 11-HPETE **12** and 15-HPETE **14**.³⁷ Whilst enzymes offer the availability of all regioisomers of HPETE **12-14** and **44-46** in greater yields than *via* autoxidation, only soybean 15-LO is commercially available in quantities suitable for the synthesis of a single HPETE enantiomer on a milligram scale.

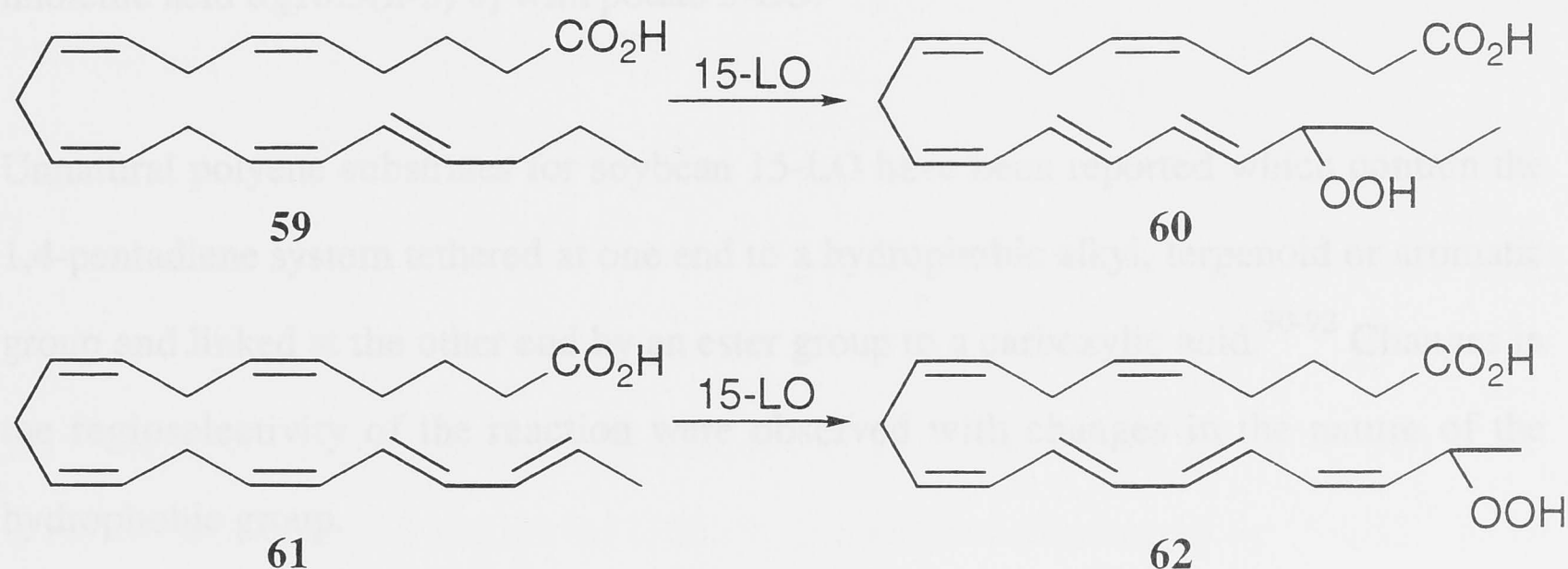
The main physiological role of mammalian LO enzymes is the regulation in levels of eicosanoids, potent inflammatory mediators derived from arachidonic acid **3**.^{4,55} With the elucidation of the structures of prostaglandins⁶⁶ and confirmation that prostaglandins and other eicosanoids form *via* arachidonic acid **3** oxidation, the investigation of LO enzymes, substrates and products in animals has become a field of intense study.⁶⁷ LO inhibitors and eicosanoid receptor antagonists have widespread applications in the treatment of inflammatory diseases such as asthma and arthritis.^{13,68,69} For example, Zileuton is a potent 5-LO inhibitor which blocks the first step in the formation of leukotrienes and has been recently approved for the treatment of asthma.⁷⁰ A second example is the antiinflammatory drug aspirin which blocks the first step in the synthesis of all prostaglandins by acetylation of the terminal amino group in the cyclooxygenase (11,15-LO) subunit of prostaglandin synthase.¹

In general LO enzymes display a tolerance towards structural changes in the substrate. For example, soybean 15-LO oxidises substrates varying in chain length from 16 to 22 carbons and varying in unsaturation from diene to hexaene.⁷¹ Many LO enzymes including 5-LO,⁷²⁻⁷⁹ 12-LO^{80,81} and 15-LO⁸² are inactivated by their own products and the products of other LO enzymes such as isomers of HPETE **12-14** and **44-46**, HETE **48-53** and leukotrienes.¹³

The inhibitory activity of hydroperoxides has been ascribed to the instability of all hydroperoxide products which can lead to irreversible damage of the enzyme active site.⁷⁹ Yet HPETE isomers,¹³ HETE isomers^{83,84} and ketones derived from HETE isomers⁸⁵ act as inhibitors of LO enzymes by acting as competitive substrates for LO or by occupying the enzyme active site. For instance, 15-HPETE **14** inhibits human polymorphonuclear leukocyte (PML) 5-LO without reacting⁷² and 15-HETE **57** inhibits human PML 5-LO by acting as a substrate to form (6*E*,8*Z*,11*Z*,13*E*)-(5*S*,15*S*)-diHETE.⁷³ The acid (9*Z*,11*E*,15*Z*)-(13*S*)-hydroperoxyoctadecatrienoic acid inhibits rat basophilic leukemia cell (RBL) 12-LO by oxidation of the enzyme active site.⁸¹

The mechanism of reaction of arachidonic acid **3** with soybean 15-LO is proposed to involve proton removal by a basic group on the enzyme from the methylene group at carbon-13 and concurrent electrophilic attack at carbon-15 by the ferric ion at the enzyme catalytic site.⁸⁶⁻⁸⁸ Oxygen insertion occurs at carbon-15 followed by proton addition to the oxygen to form (15*S*)-HPETE (*S*)-**14**. Consistent with the proposed mechanism, as shown in Scheme 1.10, 15-LO reacts with the conjugated diene and triene synthetic substrates (16*E*)-dehydroarachidonic acid **59** and (16*E*,18*E*)-bisdehydroarachidonic acid **61**. The products **60** and **62** result from proton removal from carbon-13 followed by a shift in the site of oxygen addition from carbon-15 in the formation of (15*S*)-HPETE (*S*)-**14** to carbon-17 for the product **60** and carbon-19 for the product **62**.⁸⁶

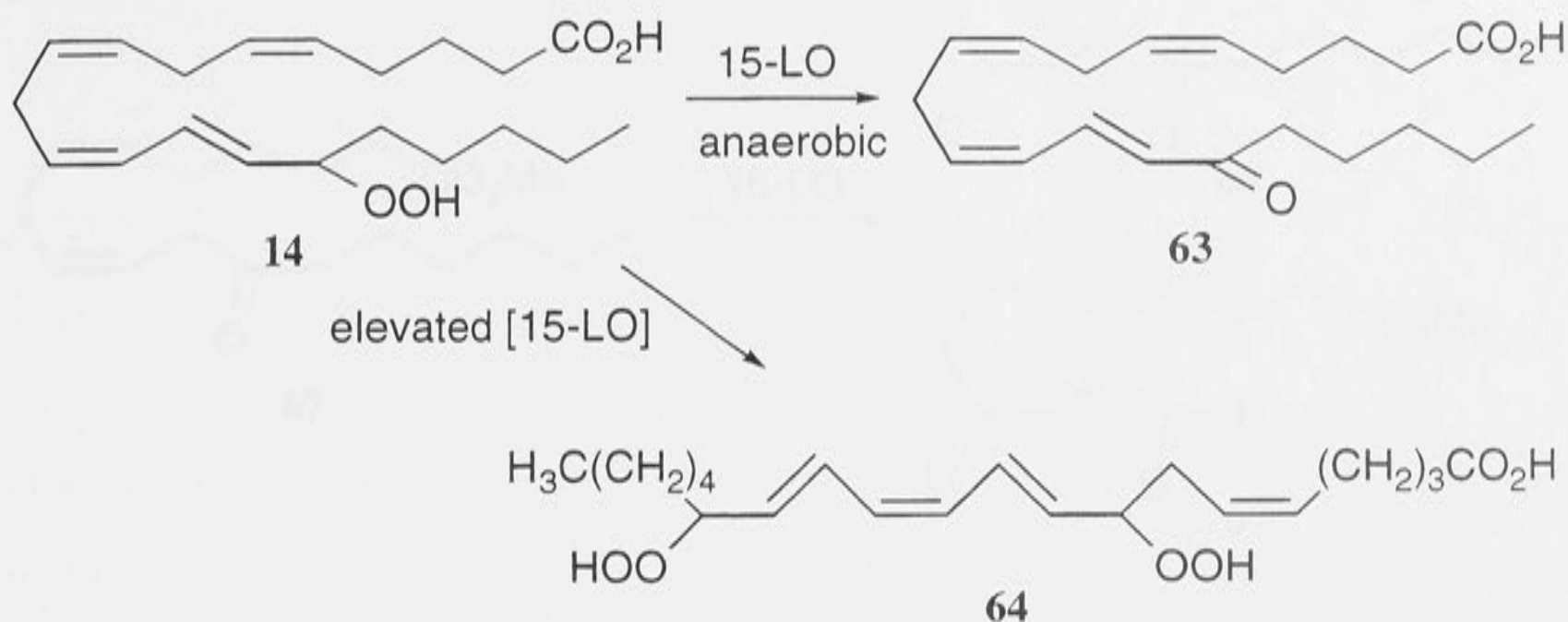
Scheme 1.10: 15-LO catalysed oxidation of the dehydroarachidonic acids **59** and **61**



Other types of transformations have been observed for LO enzymes with endogenous⁵⁴ and unnatural substrates. Mammalian 5-LO, 12-LO and 15-LO are reported to form dihydroperoxides, epoxides, ketones and aldehydes as minor products from arachidonic acid **3**.⁵⁵

Under anaerobic conditions with LO enzymes, some of the hydroperoxide formed from arachidonic acid **3** is transformed into a ketone. Shown in Scheme 1.11, 15-HPETE **14** can form 15-ketoeicosatetraenoic acid **63** (15-kETE **63**) with 15-LO and 12-HPETE **46** has also been reported to form the ketone 12-kETE with 12-LO.⁸⁹ At high concentrations of 15-LO, the triene 8,15-diHPETE **64** forms from 15-HPETE **14**,⁸³ as

Scheme 1.11 : The ketone **63** and the dihydroperoxide **64**, minor products formed using 15-LO

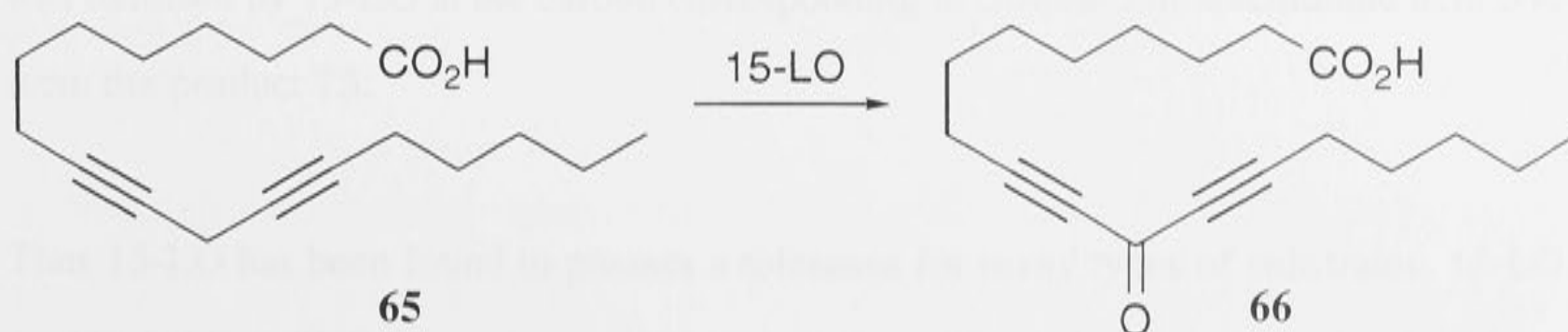


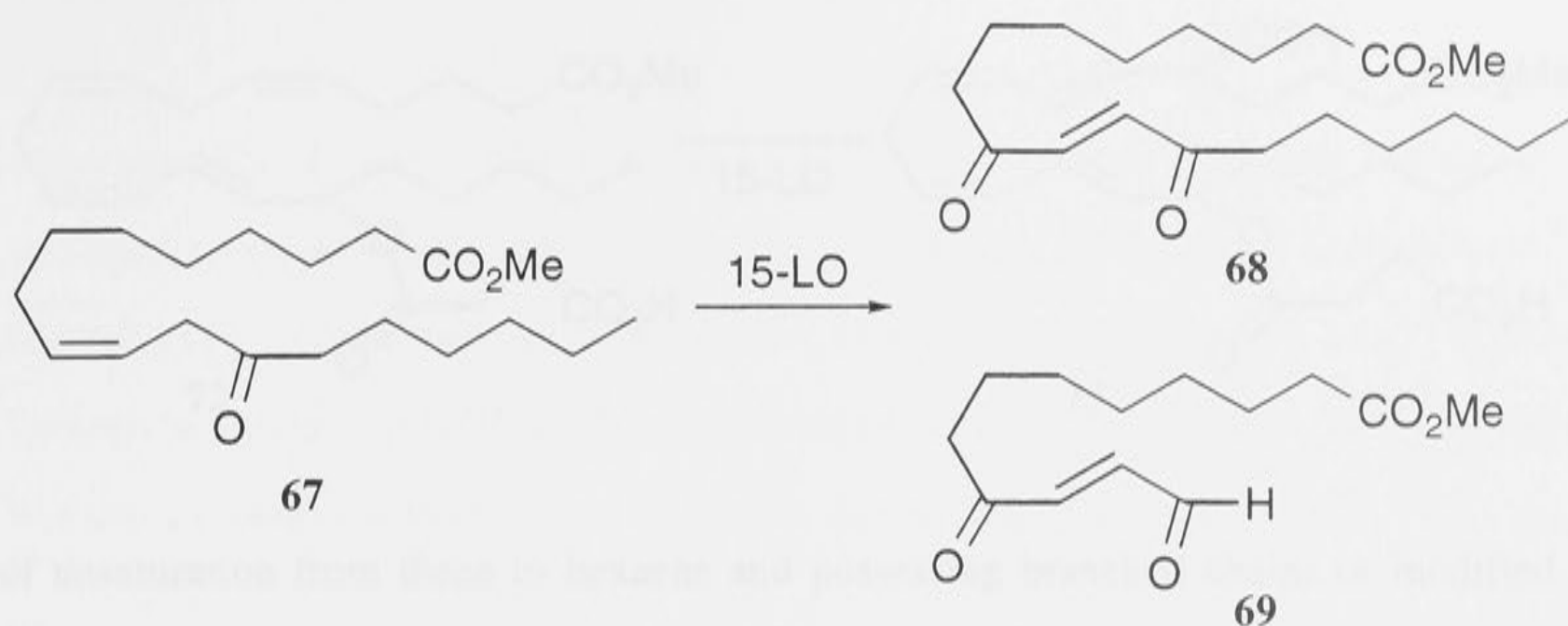
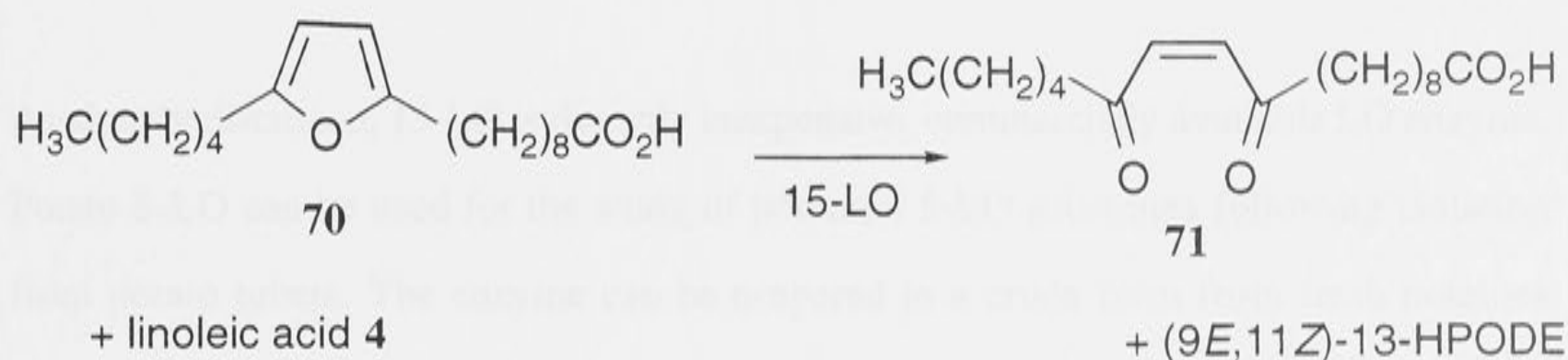
shown in Scheme 1.11 and the triene 9,16-diHPOTE is a minor product formed from linolenic acid **6** [18:3(n-3) **6**] with potato 5-LO.⁸⁴

Unnatural polyene substrates for soybean 15-LO have been reported which contain the 1,4-pentadiene system tethered at one end to a hydrophobic alkyl, terpenoid or aromatic group and linked at the other end by an ester group to a carboxylic acid.⁹⁰⁻⁹² Changes in the regioselectivity of the reaction were observed with changes in the nature of the hydrophobic group.

As shown in Schemes 1.12, 1.13 and 1.14, 15-LO has been observed to react with alkyne,⁹³ ketone and furan⁹⁴ functional groups. Octadeca-9,12-diynoic acid **65** is an irreversible inhibitor of 15-LO and is converted to 11-oxooctadeca-9,12-diynoic acid

Scheme 1.12: The alkyne **65** as a substrate for 15-LO



Scheme 1.13: The ketone **67** as a substrate for 15-LOScheme 1.14: The furan **70** as a substrate for 15-LO

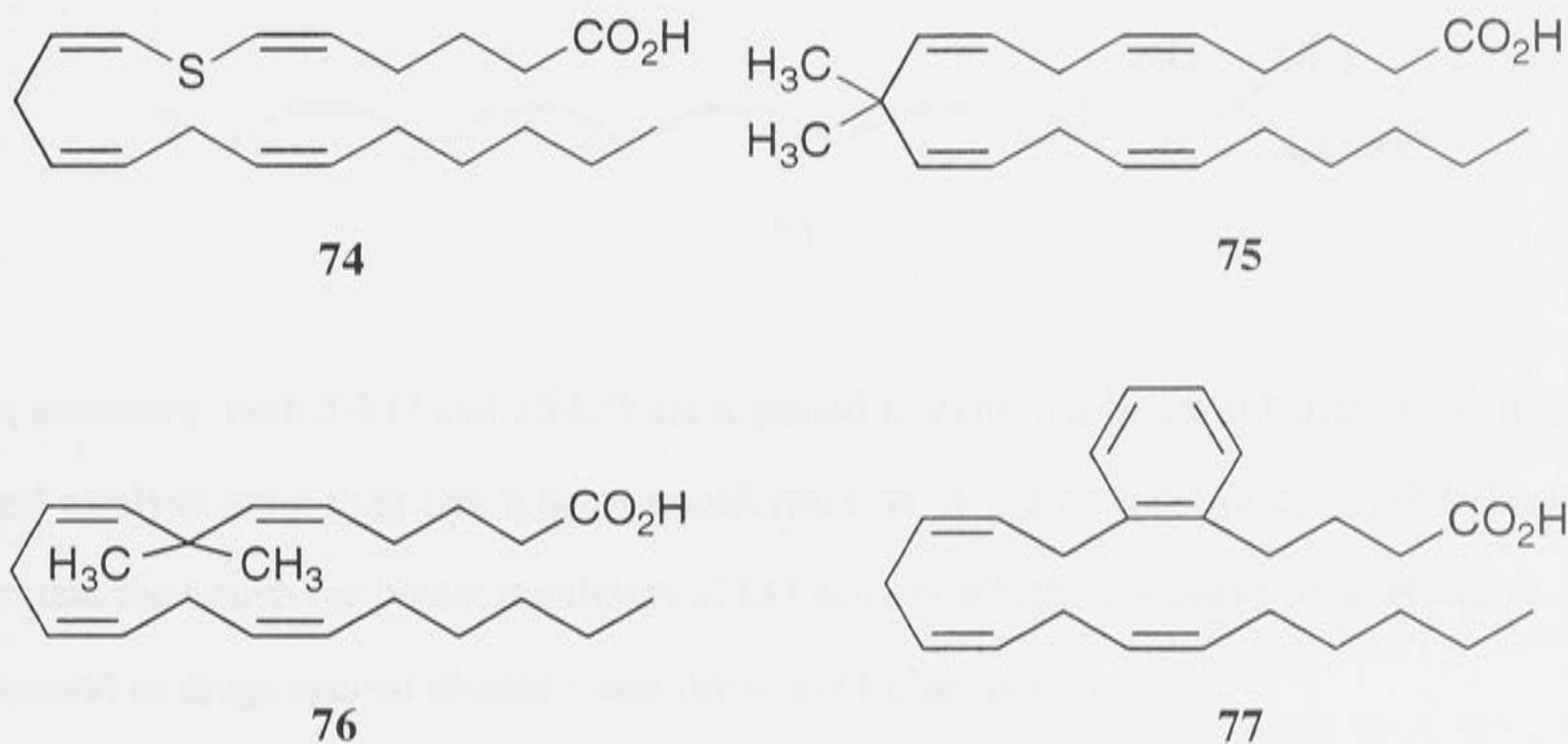
66.⁹⁵ Methyl (9Z)-12-ketooctadecenoate **67** is converted to methyl (10E)-9,12-diketooctadecenoate **68** and methyl (10E)-9,12-diketododecenoate **69** by 15-LO.⁹⁶ In the presence of linoleic acid **4** the furan fatty acid (10Z,12Z)-10,13-epoxyoctadecadienoic acid **70** is converted to (11Z)-10,13-dioxooctadecaenoic acid **71**.⁹⁴

Shown in Scheme 1.15, the regioselectivity of the 15-LO catalysed reaction has been changed using the ester (11Z,13E)-15-MeHETE and attaching an alkyl chain with a terminal carboxylic acid group to the alcohol at carbon-15.⁹² The 15-HETE derivative **72** was oxidised by 15-LO at the carbon corresponding to carbon-5 in arachidonic acid **3** to form the product **73**.

Thus 15-LO has been found to possess a tolerance for many types of substrates. 15-LO readily oxidises PUFAs varying in chain length from 16 to 22 carbons, varying in degree

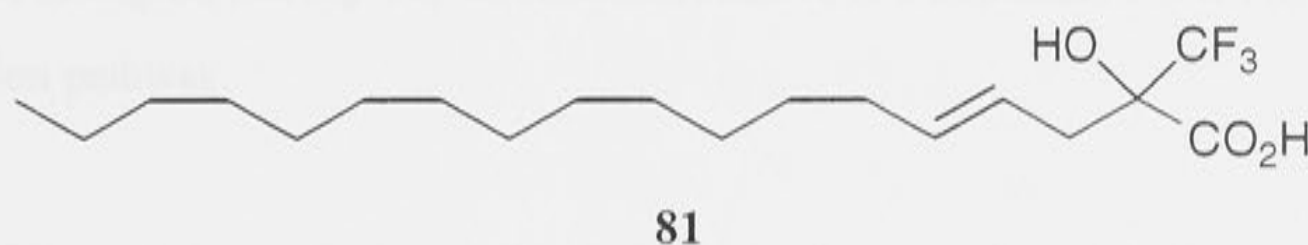
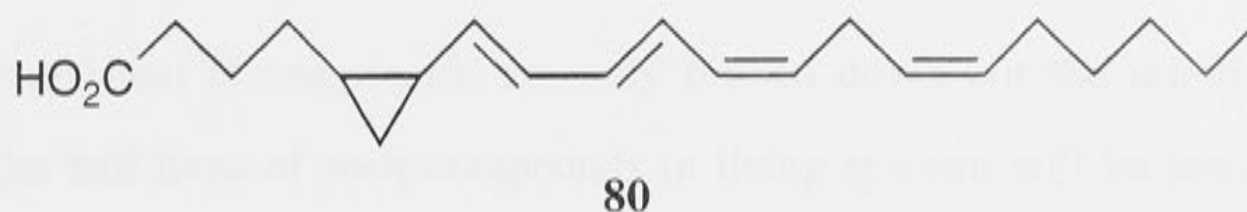
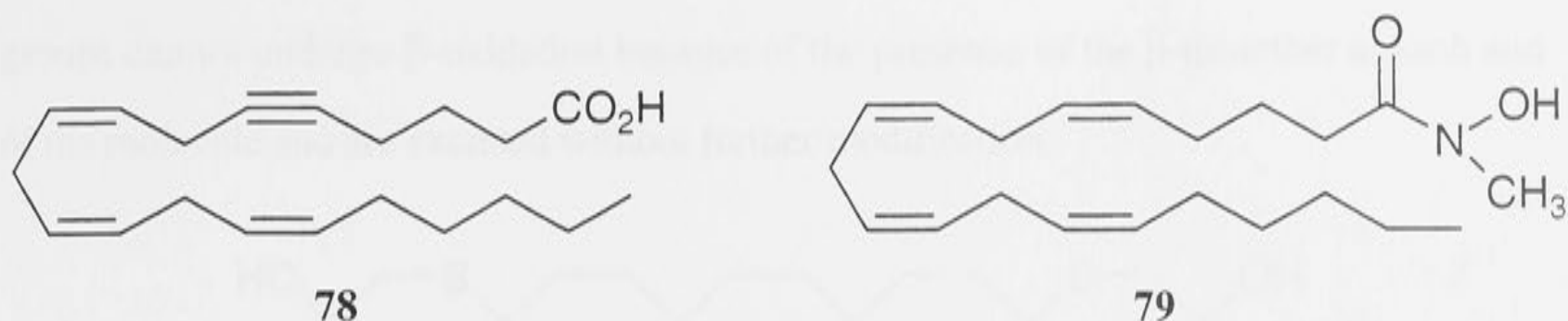
in humans and consequently could be used to treat diseases involving inflammation, such as asthma.

Many synthetic analogues of arachidonic acid **3** have been shown to regulate LO activity and amongst the examples 7-thiaarachidonic acid **74** is an irreversible 5-LO inhibitor⁹⁹ and (9*Z*,11*E*)-13-thiaoctadecadienoic acid is a competitive inhibitor of 15-LO.¹⁰⁰ 10,10-Dimethylarachidonic acid **75** and 7,7-dimethylarachidonic acid **76** promote 5-LO function whereas 5,6-benzoarachidonic acid **77** inhibits human PML 5-LO function.¹⁰¹



5,6-Dehydroarachidonate **78** irreversibly inhibits 5-LO and the *N*-methylhydroxyamide **79** derived from arachidonic acid **3** is a potent reversible inhibitor of 5-LO which inhibits the oxidation of arachidonic acid **3** by 50% at a concentration of 30 nM.¹⁰² (7*E*,9*E*,11*Z*,14*Z*)-*trans*-5,6-methanoeicosatetraenoic acid **80** inhibits PML 5-LO activity by 50% at a concentration of 3 μ M.¹⁰³

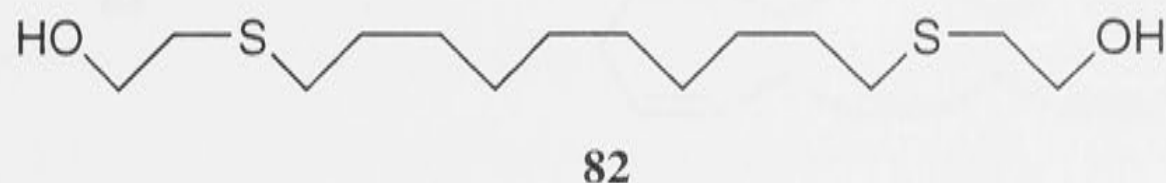
The branched monounsaturated fatty acid, (4*E*)-(R,S)-2-hydroxy-2-trifluoromethyloctadecenoic acid **81** at a concentration of 100 μ M has been reported to increase potato 5-LO activity whilst itself remaining unmodified by the enzyme and consequently binding to an allosteric regulatory centre has been proposed.¹⁰⁴



In summary, both 5-LO and 15-LO are reported to exhibit a broad substrate specificity and catalyse more than one type of transformation. A major focus of research is based around the search for potent regulators of LO activity which may prove to be effective in humans as drugs against disease states involving inflammation.

As discussed earlier, the main pathway of fatty acid breakdown involves the β -oxidation pathway (Scheme 1.1).^{1,3} In systems where the structure of a fatty acid makes β -oxidation impossible, minor routes of breakdown include α -oxidation for branched fatty acids and ω -oxidation for 2-disubstituted or 3-disubstituted branched fatty acids and for some xenobiotics.^{1,2,105} For example, alkylthioacetic acids or β -thia fatty acids cannot be metabolised by β -oxidation alone and are instead broken down by ω -oxidation and subsequent chain shortening by β -oxidation of the formerly lipophilic end of the molecule.¹⁰⁶ Alkylthioacetic acids have hypolipemic properties and induce peroxisomal β -oxidation. In humans 75% of the dose of the hypolipemic drug tiadenol **82** is excreted following oxidation of the alcohol and thioether groups as the carboxylic acid or dicarboxylic acid derivatives and sulfoxidised carboxylic acid or dicarboxylic acid derivatives.¹⁰⁷ Unlike endogenous fatty acids, the metabolites with carboxylic acid

groups cannot undergo β -oxidation because of the presence of the β -thioether at each end of the molecule and are excreted without further modification.



It is anticipated that if compounds are only broken down *via* the minor oxidation pathways, the half lives of such compounds in living systems will be longer and the associated biological activity will be sustained relative to compounds broken down *via* the β -oxidation pathway.

Within our research group, the series of PUFA analogues **83-87** has been synthesised, having a heteroatom such as sulphur or oxygen at the β -position or γ -position.^{108,109} A second synthetic series including the compounds **88** and **89** are PUFAs linked to α -amino acids as amides. Using an assay to measure one step of the β -oxidation pathway, the dehydrogenation of fatty acyl-CoA to enoyl-CoA by acyl-CoA oxidase did not occur when acyl-CoA thioesters of the synthetic compounds **83-85**, **87** and **88** were used as substrates. The amide **89** has yet to be examined. The acyl-CoA thioester of the γ -thioether **86** acted as a substrate for acyl-CoA oxidase and could act as a substrate in the β -oxidation pathway. However, the shortened acyl-CoA thioester with an α -thioether which would form *via* the β -oxidation pathway would be unable to undergo further chain shortening. Hence, the acyl-CoA thioesters of the compounds **83-89** are unsuitable for complete metabolism *via* the β -oxidation pathway and consequently will have longer half lives in biological systems than endogenous PUFAs.

The synthetic compounds **83-89** are biologically active and have been shown to effect cytokine production by leukocytes and neutrophil function *in vitro* as well as being regulators of inflammation *in vivo* using the mouse paw oedema model.^{110, 111}



83



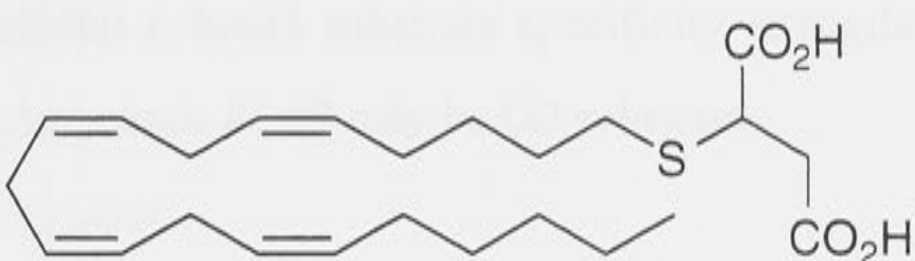
84



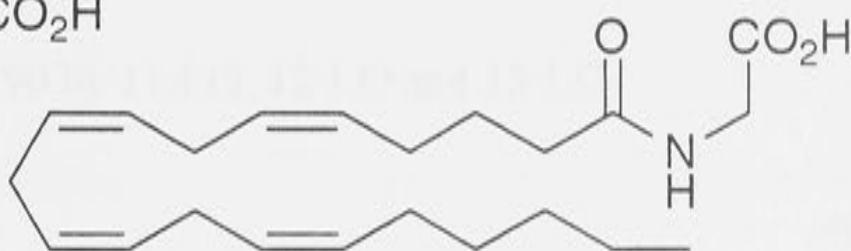
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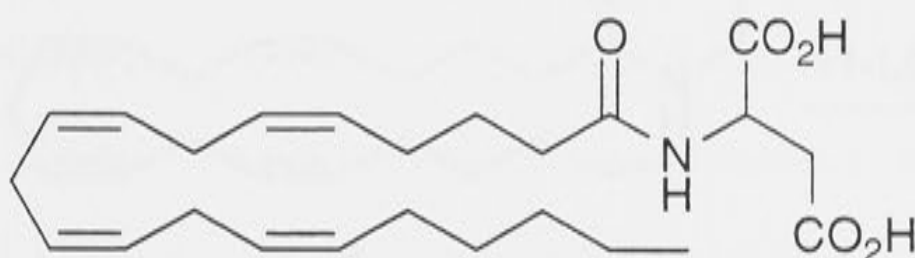
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88



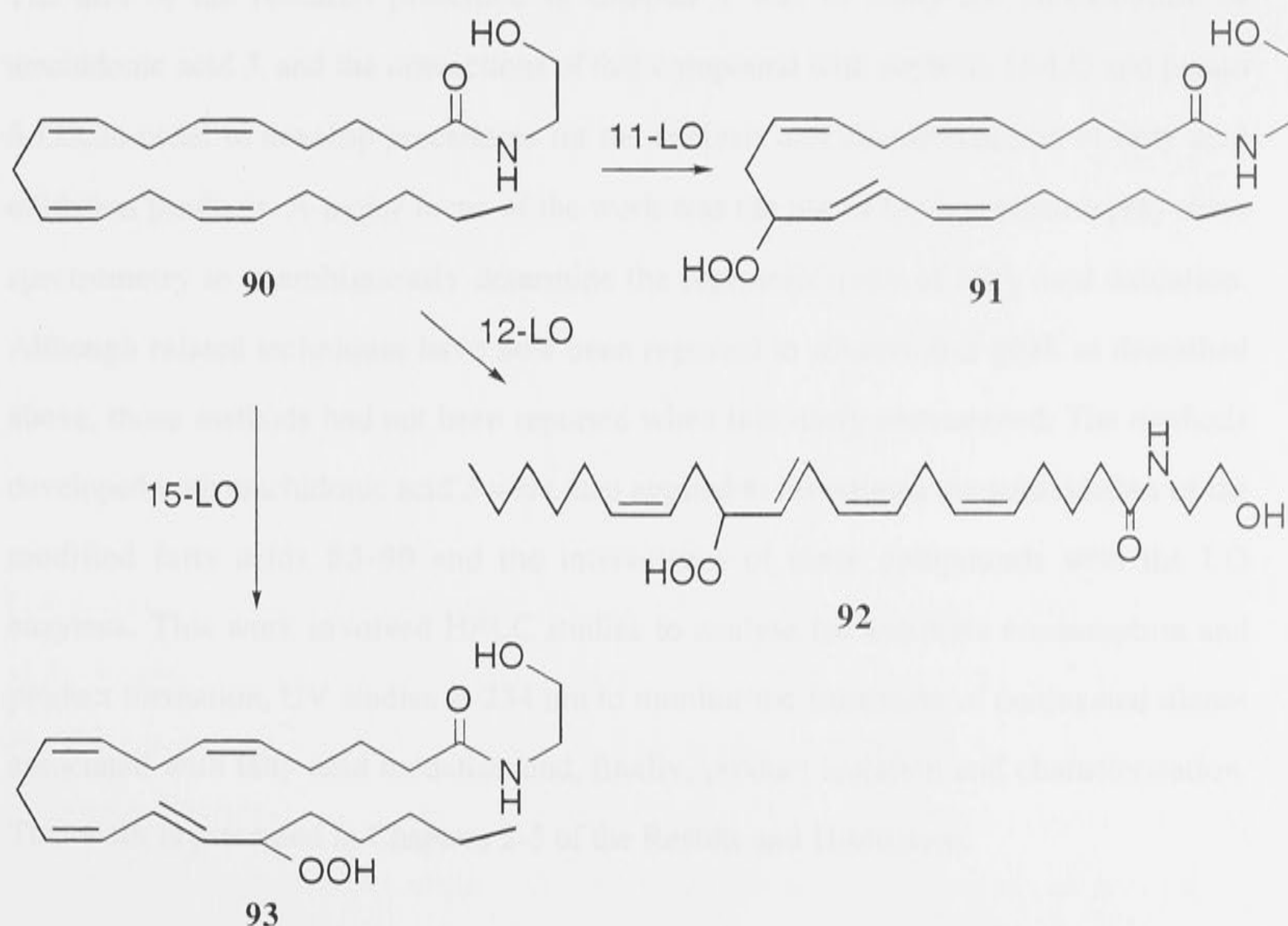
89

Shown in Scheme 1.16, anandamide **90** (arachidonate ethanolamide **90**) is an eicosanoid identified in porcine brain lipids and in 1992 was reported to be the first known endogenous ligand to bind to the brain cannabinoid receptor with a receptor binding affinity comparable to that of the major psychoactive ingredient of marihuana, Δ^9 -tetrahydrocannabinol.¹¹² Structurally, the amide **88** differs only slightly from anandamide **90** by having a carboxylic acid group in place of the alcohol group at carbon-1.

In 1995 anandamide **90** was shown to be a substrate for leucocyte 11-LO, brain 12-LO and soybean 15-LO and the products 11-hydroperoxyanandamide **91** (11-HPANA **91**), 12-HPANA **92** and 15-HPANA **93**, respectively, were derivatised and characterised by mass spectrometry.⁴⁶ In a receptor binding assay for the brain cannabinoid receptor 12-hydroxyanandamide (12-HANA) was found to have twice the receptor affinity of anandamide **89** and was proposed to be a modulator of neuronal function.

As anandamide **90** analogues, the amides **88** and **89** and their metabolites are potentially active as brain lipids. The likeness in the structures of the endogenous compound anandamide **90** and the synthetic amide **88** coupled with the knowledge that LO enzymes exhibit a broad substrate specificity strengthens the hypothesis that the synthetic compounds **83-89** may be LO substrates.

Scheme 1.16: The oxidation of anandamide **90** by 11-LO, 12-LO and 15-LO



As discussed earlier, feedback inhibition of LO activity by fatty acid oxidation products has been documented. As analogues of arachidonic acid **3**, in living systems the synthetic compounds **83-89** have the potential to undergo many of the enzyme catalysed processes leading to eicosanoids and consequently to interfere with the function of eicosanoids and the enzymes involved in eicosanoid formation. As already discussed and shown in Fig 1.1, mixtures of autoxidised PUFAs display enhanced biological properties compared to individual PUFAs. Hence, it is likely that the oxidation products derived from the synthetic compounds **83-89** will be biologically active.

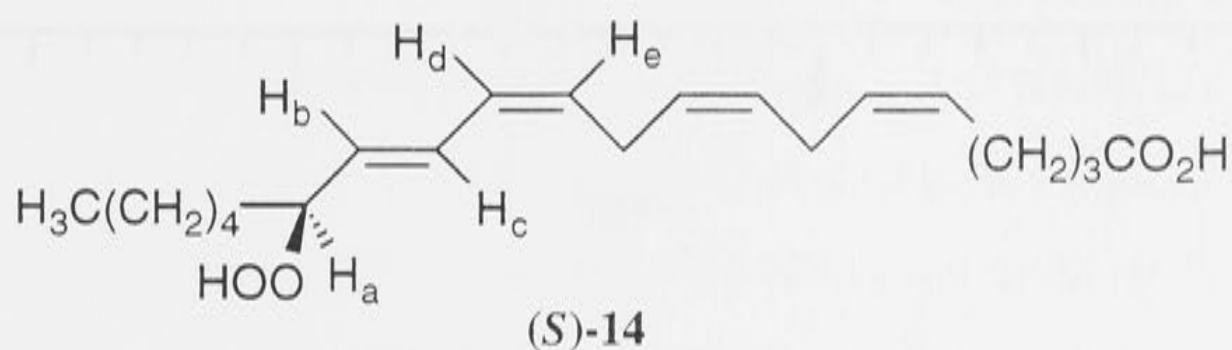
The theme of the research presented in the Results and Discussion is the development and application of techniques for the preparation, isolation and characterisation of fatty acid oxidation products derived from chemical mediated oxidation and enzyme catalysed oxidation.

The aim of the research presented in Chapter 1 was to study the autoxidation of arachidonic acid **3**, and the interactions of that compound with soybean 15-LO and potato 5-LO, in order to develop procedures for the analysis and characterisation of fatty acid oxidation products. A major focus of the work was the use of tandem electrospray mass spectrometry to unambiguously determine the regioselectivity of fatty acid oxidation. Although related techniques have now been reported to achieve this goal, as described above, those methods had not been reported when this study commenced. The methods developed with arachidonic acid **3** were then applied to investigate the autoxidation of the modified fatty acids **83-89** and the interactions of these compounds with the LO enzymes. This work involved HPLC studies to analyse for substrate consumption and product formation, UV studies at 234 nm to monitor the formation of conjugated dienes associated with fatty acid oxidation and, finally, product isolation and characterisation. That work is presented in Chapters 2-5 of the Results and Discussion.

Synthesis and characterisation of oxidised derivatives of natural fatty acids

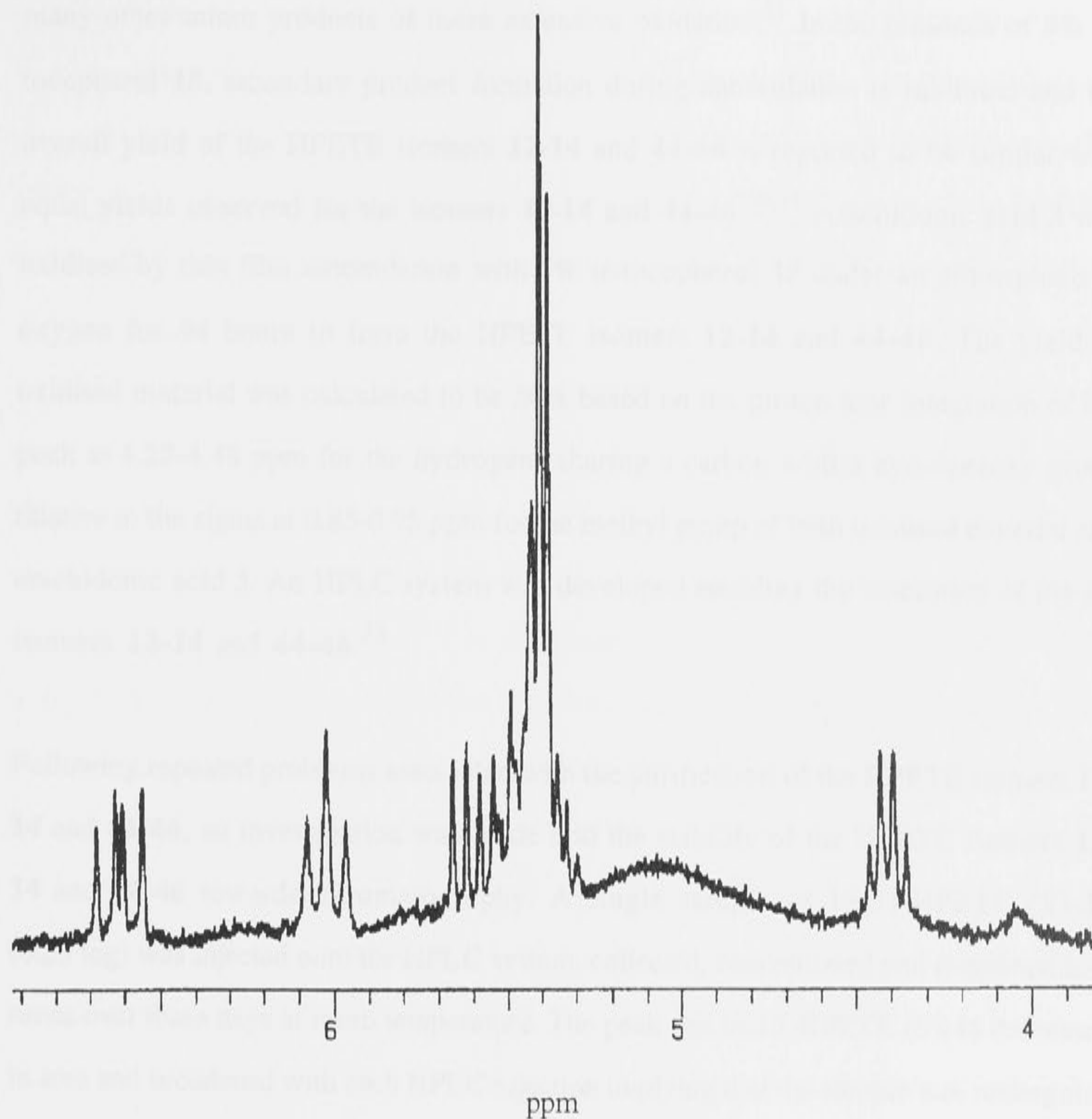
As discussed in the Introduction, the initial goal of this project was to investigate techniques for the preparation, isolation and characterisation of the primary products of arachidonic acid **3** autoxidation, the HPETE regioisomers **12-14** and **44-46**.

(15*S*)-HPETE (**S**)-**14** was prepared from arachidonic acid **3** as a standard for spectroscopy and chromatography using the commercially available enzyme soybean 15-LO in buffer. The compound (**S**)-**14** was isolated by extraction into dichloromethane and purified by preparative scale thin layer chromatography (TLC). In the proton nmr spectra, several signals made it possible to distinguish between (15*S*)-HPETE (**S**)-**14** and arachidonic acid **3**. As shown in Fig 2.1, the olefinic protons of the product (**S**)-**14** are observed as a multiplet at 5.35-5.49 ppm and as signals at 5.60, 6.02 and 6.60 ppm in the area ratio 5 : 1 : 1 : 1. The corresponding eight olefinic protons in arachidonic acid **3** are observed as a single broad multiplet at 5.28-5.51 ppm. The signal due to the hydrogen sharing carbon-15 with the hydroperoxide group is observed as a doublet of triplets at 4.40 ppm.



The stereochemistry of the conjugated double bonds can be confirmed based on the value of the vicinal coupling constants ($^3J_{HH}$) because $^3J_{HH}$ is 0-12 Hz within a *Z*-alkene and 12-18 Hz within an *E*-alkene. Between the hydrogens neighbouring the *E*-double bond, attached to carbon-13 (H_c , 6.60 ppm) and carbon-14 (H_b , 5.60 ppm), $^3J_{bc}$ equals 15.0 Hz. Between the hydrogens neighbouring the *Z*-double bond, attached to carbon-11 (H_e , 5.35-5.49 ppm) and carbon-12 (H_d , 6.02 ppm), $^3J_{de}$ equals 11.0 Hz.

Fig 2.1: The ^1H nmr spectrum of (15*S*)-HPETE (**S**)-**14** between 3.9 and 6.9 ppm



In comparison with the proton nmr spectrum of 15-HETE **53** between 4.0 and 7.0 ppm, shown in Fig 1.2, the number of signals and the splitting patterns shown for (15*S*)-HPETE (**S**)-**14** in Fig 2.1 are the same but the chemical shift values make it possible to distinguish between the compounds (**S**)-**14** and **53**. The chemical shift value for the hydrogen attached to carbon-15 is 4.40 ppm for (15*S*)-HPETE (**S**)-**14** and 4.23 ppm for 15-HETE **53** and the value for the hydrogen attached to carbon-14 is 5.60 ppm for (15*S*)-HPETE (**S**)-**14** and 5.71 ppm for 15-HETE **53**.

As discussed earlier, thin film autoxidation of arachidonic acid **3** results in the formation of the HPETE isomers **12-14** and **44-46** as the principal oxidation products amongst many other minor products of more extensive oxidation.²¹ In the presence of 5% α -tocopherol **15**, secondary product formation during autoxidation is inhibited and the overall yield of the HPETE isomers **12-14** and **44-46** is reported to be similar with equal yields observed for the isomers **12-14** and **44-46**.^{30,31} Arachidonic acid **3** was oxidised by thin film autoxidation with 5% α -tocopherol **15** under an atmosphere of oxygen for 94 hours to form the HPETE isomers **12-14** and **44-46**. The yield of oxidised material was calculated to be 20% based on the proton nmr integration of the peak at 4.28-4.48 ppm for the hydrogens sharing a carbon with a hydroperoxy group relative to the signal at 0.85-0.95 ppm for the methyl group of both oxidised material and arachidonic acid **3**. An HPLC system was developed enabling the resolution of the six isomers **12-14** and **44-46**.³²

Following repeated problems associated with the purification of the HPETE isomers **12-14** and **44-46**, an investigation was made into the stability of the HPETE isomers **12-14** and **44-46** towards chromatography. A single sample of 15(*S*)-HPETE (*S*)-**14** (0.25 mg) was injected onto the HPLC system, collected, concentrated and reinjected four times over three days at room temperature. The peak due to 15-HPETE (*S*)-**14** decreased in area and broadened with each HPLC injection implying that the sample was undergoing decomposition. Polymeric material, insoluble in the HPLC solvent (hexane : isopropanol : acetic acid; 986 : 13 : 1), also formed during the procedure. Other workers have confirmed that hydroperoxy-polyunsaturated fatty acids are unstable and consequently these compounds are commonly reduced to hydroxy fatty acids prior to HPLC analysis.^{32,35,36}

An investigation was made into the instability of (15*S*)-HPETE (*S*)-**14** in solution. As (10*E*,12*Z*)-9-MeHPODE **20** is known to isomerise to (9*E*,11*Z*)-13-MeHPODE **21**, (10*E*,12*E*)-9-MeHPODE **22** and (9*E*,11*E*)-13-MeHPODE **23**,³⁶ an attempt was made to observe isomerisation of 15-HPETE (*S*)-**14** under similar conditions. 15-HPETE (*S*)-

14 in hexane (20 mg / ml) was stored at 40 °C (0, 24 and 48 hours) and samples were analysed by HPLC after reduction to convert hydroperoxides to the more stable alcohols using sodium borohydride. As 15-HETE **53** was the only isomer observed it was concluded that whilst decomposition occurs as shown above, no isomerisation had taken place. On the basis of the reports and experimental observations of the instability of fatty acid hydroperoxides, it was concluded that the hydroxyeicosatetraenoic acid (HETE) isomers **48-53** would be more appropriate candidates for analysis and characterisation.

An HPLC assay was developed to establish the optimum conditions for the formation of the HETE isomers **48-53** from arachidonic acid **3** by autoxidation in the presence of various amounts of α -tocopherol **15**, followed by reduction. Published HPLC assays cannot be used to completely resolve all six HETE isomers **48-53** making them unsuitable as analytical assays.^{32,34} In particular, using unmodified silica 15-HETE **53** and 12-HETE **52** have similar retention times under the published conditions. The polarity of the mobile phase in the unmodified silica HPLC system used above for 15-HPETE (**S**)-**14** was decreased to hexane : isopropanol : acetic acid in the ratio 1000 : 7 : 1 and the flow rate was halved. Then 11-HETE **51**, 12-HETE **52** and 15-HETE **53** were resolved with retention times [R_t] of 24, 18 and 20 minutes, respectively. Consequently, the retention times of the other three isomers 5-HETE **48**, 8-HETE **49** and 9-HETE **50** were between 30 and 70 minutes. The isomer of highest retention time, 5-HETE **48**, readily undergoes lactonisation during sample preparation resulting in a relatively small or absent peak for this compound by HPLC.

Salicylic acid (R_t = 16 minutes) was used as an external standard in the HPLC assay and enabled the comparison of yields of the isomers **51-53** under various conditions. After each 30 minute analytical run the HPLC column was rinsed for five minutes with a more polar mobile phase and allowed to equilibrate with the first mobile phase for ten minutes before the analysis of the next sample.

Whilst the molecules are not actually symmetrical, because of the symmetry of the octatriene and undecatetraene systems of linolenic acid **6** and arachidonic acid **3**, respectively, pairs of symmetrically related isomers form in similar yields. From the Introduction, the symmetrically related pairs of MeHOTE formed from methyl linolenate **38** by autoxidation followed by reduction to alcohols are 9-MeHOTE and 16-MeHOTE, and 12-MeHOTE and 13-MeHOTE. (10*E*,12*Z*,15*Z*)-9-MeHOTE and (9*Z*,12*Z*,14*E*)-16-MeHOTE form in the ratio 27 : 31, (9*Z*,13*E*,15*Z*)-12-MeHOTE and (9*Z*,11*E*,15*Z*)-13-MeHOTE form in the ratio 6 : 7, and the geometric isomers of the same regioisomers (10*E*,12*E*,15*Z*)-9-MeHOTE and (9*Z*,12*E*,14*E*)-16-MeHOTE form in the ratio 11 : 13, and (9*Z*,13*E*,15*E*)-12-MeHOTE and (9*E*,11*E*,15*Z*)-13-MeHOTE form in the ratio 2 : 3.²² For methyl linolenate **38** the yield of each isomer in a pair of symmetrically related regioisomers is approximately equal *via* autoxidation with no additive and (10*E*,12*Z*,15*Z*)-9-MeHOTE, (9*Z*,13*E*,15*Z*)-12-MeHOTE, (9*Z*,11*E*,15*Z*)-13-MeHOTE and (9*Z*,12*Z*,14*E*)-16-MeHOTE are the principal MeHOTE isomers formed in nearly equal amounts in the presence of a 5-10% by weight concentration of α -tocopherol **15**.

The same phenomenon of symmetrically related isomers forming in nearly equal yields was anticipated for arachidonic acid **3**. The pairs of symmetrically related HETE regioisomers assumed to form in equal amounts are 5-HETE **48** and 15-HETE **53**, 8-HETE **49** and 12-HETE **52**, and 9-HETE **50** and 11-HETE **51**. Hence, the ratio of conjugated *E,Z*-diene containing isomers observed for 11-HETE **51**, 12-HETE **52** and 15-HETE **53** by HPLC is proposed to be approximately equal to the ratio of the isomers 9-HETE **50**, 8-HETE **49** and 5-HETE **48**, and for this reason only the former three isomers **51-53** were monitored.

The solvent was evaporated from a solution of arachidonic acid **3** and α -tocopherol **15** in dichloromethane leaving a thin film which was stored under oxygen for 72 hours. At the completion of autoxidation, triphenylphosphine in dichloromethane was added to the flask to form the HETE isomers **48-53**. The dichloromethane was evaporated under nitrogen, the residue was resuspended in the HPLC solvent and the standard, salicylic acid, was

added to the mixture. The ratio of (12*E*,14*Z*)-11-HETE **51**, (8*Z*,10*E*)-12-HETE **52** and (11*E*,13*Z*)-15-HETE **53** was recorded by HPLC. The yield of the HETE isomers **51-53** was calculated based on the extinction coefficients of HETE and salicylic acid. The results are shown in Table 2.1.

Table 2.1: Yields of the HETE isomers **51-53** formed by autoxidation

% α -Tocopherol 15	11-HETE 51	12-HETE 52	15-HETE 53
Control 0%	0.4%	0.4%	1.4%
α -Toc 0.01%	< 0.01%	0.04%	0.08%
α -Toc 0.1%	0.2%	0.2%	0.6%
α -Toc 1%	1.3%	1.5%	1.6%
α -Toc 5%	5.5%	5.0%	4.4%

The mechanism of autoxidation has been discussed in detail in the Introduction (Schemes 1.3 and 1.5). The first propagation step of autoxidation involves hydrogen abstraction from arachidonic acid **3** to form a pentadienyl radical and requires a radical such as a peroxy radical or an α -tocopheroxy radical **24**. The next step of lipid oxidation involves oxygen addition to the resonance stabilised pentadienyl radical. In the subsequent propagation step hydrogen adds to the peroxy radical to form one of the HPETE isomers **12-14** and **44-46** and requires a hydrogen donor, such as arachidonic acid **3** or α -tocopherol **15**. Reduction of the hydroperoxides **12-14** and **44-46** to the alcohols **48-53** using triphenylphosphine is assumed to be quantitative. In Table 2.1, following arachidonic acid **3** autoxidation and reduction, the yields of the HETE isomers **51-53** are clearly influenced by the concentration of α -tocopherol **15** during the autoxidation step.

A higher yield of 15-HETE **53** relative to 11-HETE **51** and 12-HETE **52** is observed in the control sample and at low concentrations of α -tocopherol **15**. The peroxy radical precursors of 11-HETE **51** and 12-HETE **52** may undergo competing 1,5-*exo* cyclisation but the peroxy radical precursor of 15-HETE **53** is unable to do so. As the

concentration of the hydrogen donor α -tocopherol **15** is increased, the ratio of the isomers becomes uniform because the rate of hydrogen addition to form the hydroperoxides **12**, **14** and **46** from the peroxy radicals is higher than the rate of 1,5-*exo* cyclisation. The effect of α -tocopherol **15** on the yields of the HPETE isomers **12**, **14** and **46** by autoxidation is reflected in the yields of the alcohols **51-53** following reduction of the autoxidation products.

At low concentrations of α -tocopherol **15** (0.01% and 0.1%), the lower yields of the HETE isomers **51-53** relative to the control sample confirm that α -tocopherol **15** acts as an antioxidant. At these concentrations α -tocopherol **15** inhibits the oxidation process by acting as a hydrogen donor to trap peroxy radicals or alkyl radicals before they propagate the oxidation process.

At elevated α -tocopherol **15** concentrations, the antioxidant **15** rapidly donates hydrogen to peroxy radical intermediates trapping them as the HPETE isomers **12-14** and **44-46** in close to uniform quantities by inhibiting the decomposition of peroxy radicals *via* 1,5-*exo* cyclisation. Hence, a uniform yield of the HETE isomers **51-53** is observed. At elevated α -tocopherol **15** concentrations the yield of each of the hydroperoxides **12**, **14** and **46** observed as the alcohols **51-53** has also increased so that the rate of hydroperoxide formation appears to have increased. This rate increase is described as the 'prooxidant effect' of α -tocopherol **15**.¹¹³⁻¹¹⁵

The total yield of the HETE isomers **51-53** is 14.9% and assuming the other three symmetrically related HETE isomers **48-50** form in a similar yield, the total yield of the HETE isomers **48-53** is about 30%. Relative to the control sample, the 'prooxidant' concentrations (1-5%) of α -tocopherol **15** result in uniform and elevated yields of the HPETE isomers **12-14** and **44-46** or the HETE isomers **48-53** following reduction of the autoxidation products. In this study, following autoxidation for 72 hours, the total yield of the HETE isomers **51-53** is over six times higher using 5% α -tocopherol **15** relative to the control sample and the yield of every isomer has increased by more than

three times. In contrast with this result, in studies of the autoxidation of the linoleate esters **16** and **33** over the same period, it has been reported that whilst a 5-10% by weight concentration of α -tocopherol **15** increases the yield of the hydroperoxide isomers containing conjugated *E,Z*-diene groups, the total yield of monohydroperoxides decreases by 20-50%.^{25,27}

The increase in the yields of the HETE isomers **51-53** formed by arachidonic acid **3** autoxidation with α -tocopherol **15** and subsequent reduction may also be possible using other compounds which interfere with the oxidation process. The experiment outlined above was repeated with the α -tocopherol **15** analogue, butylated hydroxytoluene **93** (BHT **93**), the polar antioxidant ascorbic acid **94** (commonly known as vitamin C **94**) and the thiol antioxidant glutathione **95**. The results for the autoxidation experiments with the antioxidants **93**, **94** and **95** are shown in Tables 2.2, 2.3 and 2.4.

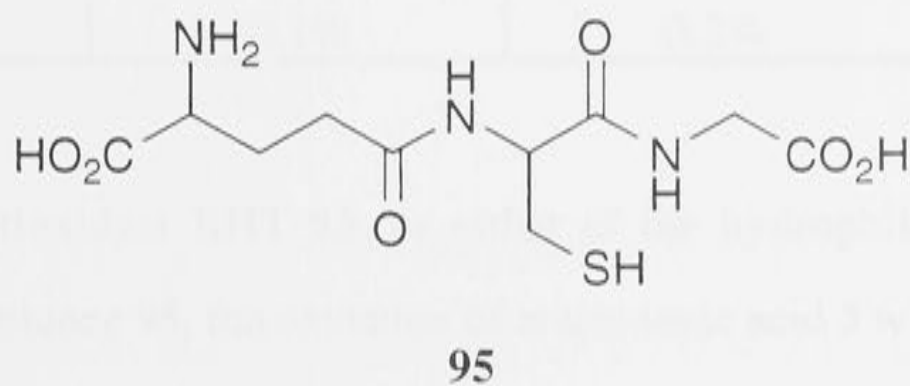
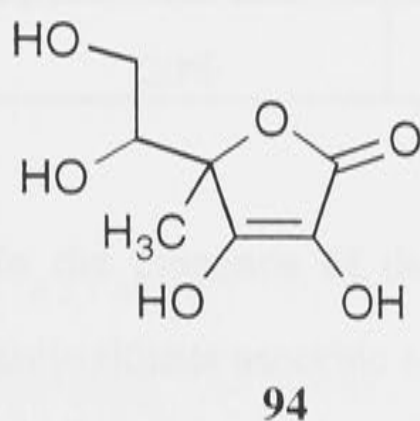
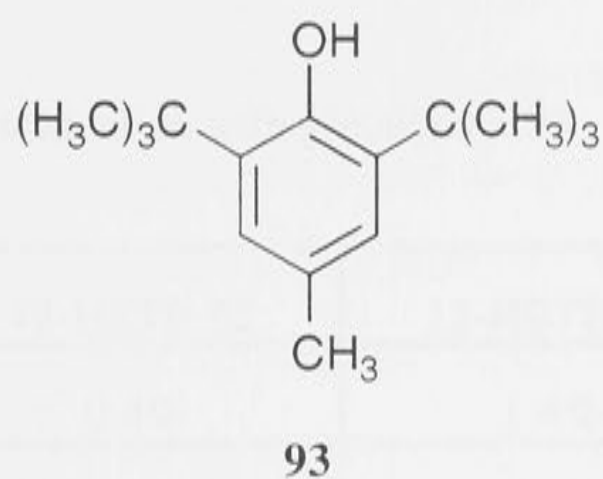
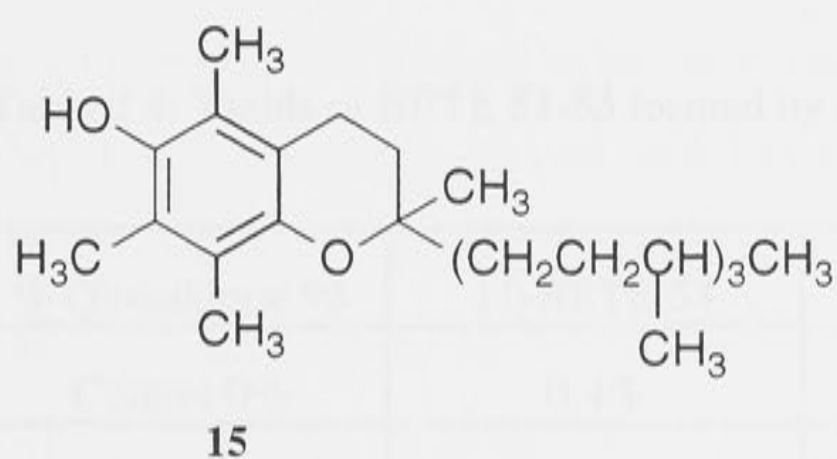


Table 2.2: Yields of the HETE isomers **51-53** formed by autoxidation with BHT **93**

% BHT 93	11-HETE 51	12-HETE 52	15-HETE 53
Control 0%	0.4%	0.4%	1.4%
0.01%	0.02%	0.02%	0.03%
5%	< 0.01%	< 0.01%	< 0.01%
20%	< 0.01%	0.01%	< 0.01

Table 2.3: Yields of the HETE isomers **51-53** formed by autoxidation with vitamin C **94**

% Ascorbic acid 94	11-HETE 51	12-HETE 52	15-HETE 53
Control 0%	0.4%	0.4%	1.4%
0.01%	0.03%	0.02%	0.02%
5%	0.04%	0.04%	0.2%
20%	0.03%	0.04%	0.2%

Table 2.4: Yields of HETE **51-53** formed by autoxidation with glutathione **95**

% Glutathione 95	11-HETE 51	12-HETE 52	15-HETE 53
Control 0%	0.4%	0.4%	1.4%
0.01%	0.04%	0.01%	0.02%
5%	0.04%	0.03%	0.1%
20%	0.2%	0.1%	0.2%

In the presence of the lipophilic antioxidant BHT **93**, or either of the hydrophilic antioxidants ascorbic acid **94** and glutathione **95**, the oxidation of arachidonic acid **3** was inhibited compared to the control sample. The lowest overall yields of the HETE isomers **51-53** observed with the polar antioxidants **94** and **95** were about ten times the lowest yield observed for BHT **93**. Hence, BHT **93** was most efficient at inhibiting formation of the HPETE isomers **12**, **14** and **46** by autoxidation. For all three antioxidants **93**, **94**

and **95**, the yield of 15-HETE **53** was consistently higher than the yields of either 12-HETE **52** or 11-HETE **51**. As already discussed, the peroxy radical precursors of 12-HETE **52** and 11-HETE **51** are able to decompose *via* 1,5-*exo* cyclisation and consequently the yields of these isomers formed from the HPETE isomers **12** and **46** are lower than the yield of 15-HETE **53**. In conclusion, prooxidant activity was only observed using 1-5% α -tocopherol **15**.

A second method for the preparation of the HETE isomers **48-53** from arachidonic acid **3** involves photolysis in methanol with a photosensitiser, methylene blue, followed by reduction.^{32,33} Following the published procedure, arachidonic acid **3** was oxidised by photolysis with methylene blue in methanol and oxygen was slowly bubbled through the solution for 16 hours. By proton nmr spectroscopy and TLC it was clear that a complex mixture of many UV active oxidised products had formed. Based on the integration of proton nmr spectroscopy peaks as outlined earlier, the yield of monohydroperoxides was calculated to be 40%.

In the literature, the primary oxidation products formed using this technique have been identified as the HETE isomers **48-53** as well as a low yield of the unconjugated 6-HETE **56** and the 14-HETE isomer which are inseparable from 5-HETE **48** and 15-HETE **53**, respectively, by HPLC.³² In agreement with the published yield, the overall yield of HETE isomers by this technique was found to be higher than for the autoxidation procedure but the inability to conveniently separate all of the HETE regioisomers makes this technique unsuitable for the isolation and characterisation of all six of the HETE regioisomers **48-53**.

(15*S*)-HETE (*S*)-**53** was prepared and characterised for subsequent use as an HPLC standard. The compound (*S*)-**53** formed from arachidonic acid **3** using soybean 15-LO followed by reduction of the crude (15*S*)-HPETE (*S*)-**14** to (15*S*)-HETE (*S*)-**53** with triphenylphosphine. (15*S*)-HETE (*S*)-**53** was purified by flash chromatography and characterised by ¹H nmr and ¹³C nmr spectroscopy. As already discussed and shown in

Fig 1.2, the HETE **53** can be distinguished from arachidonic acid **3** by a multiplet at 4.23 ppm for the hydrogen attached to carbon-15 and three signals at 5.71, 6.00 and 6.57 ppm corresponding to the vinylic protons at carbon-14, carbon-12 and carbon-13, respectively. In the ^{13}C nmr spectrum of (15*S*)-HETE (*S*)-**53**, a distinct signal is observed for carbon-15 at 72.5 ppm as well as a new set of signals for the six vinylic carbons. 15-HETE **53** can be distinguished from 15-HPETE **14** by the slight decrease in the chemical shift of the signal for the hydrogen at carbon-15 from 4.41 ppm for 15-HPETE **14** to 4.23 ppm for 15-HETE **53**.

An HPLC system was established with (15*S*)-HETE (*S*)-**53** for the separation of the regioisomers of HETE **48-53** using the solvent system hexane : isopropanol : acetic acid, in the ratio 986 : 13 : 1. Under the prooxidant conditions identified in earlier experiments, the thin film autoxidation of arachidonic acid **3** was employed to prepare the HETE isomers **49-53**. Microgram quantities of five of the six *E,Z*-conjugated diene isomers of HETE **49-53** were isolated by autoxidation of arachidonic acid **3** (25 mg) with α -tocopherol **15** (10% by weight) for 42 hours, derivatisation of the reaction mixture with sodium borohydride (1.6 mol equivalents) and isolation of the HETE isomers **49-53** using HPLC. Reinjection of each HPLC fraction confirmed that the individual isomers 15-HETE **53**, 12-HETE **52**, 11-HETE **50**, 9-HETE **49** and 8-HETE **48** were isolated in each case with retention times of 13.8, 12.5, 19.0, 26.8 and 32.5 minutes, respectively, as shown in Figs 2.2-2.6. A small amount of 12-HETE **52** (less than 1%) was observed in the fraction containing 15-HETE **53** in Fig 2.3. Based on literature reports, a peak corresponding to 5-HETE **48** would be expected to have a retention time around 50-60 minutes but was not observed. 5-HETE **48** readily forms a δ -lactone which proved difficult to recover because it is relatively nonpolar and is reported to elute by HPLC with a similar retention time to arachidonic acid **3**.

Proton nmr spectroscopy was used to confirm that the five compounds isolated by HPLC were the HETE isomers **49-53**. In each case, signals were recorded corresponding to the hydrogen (H_a) sharing a carbon with an alcohol group and the vinylic hydrogens H_b - H_e

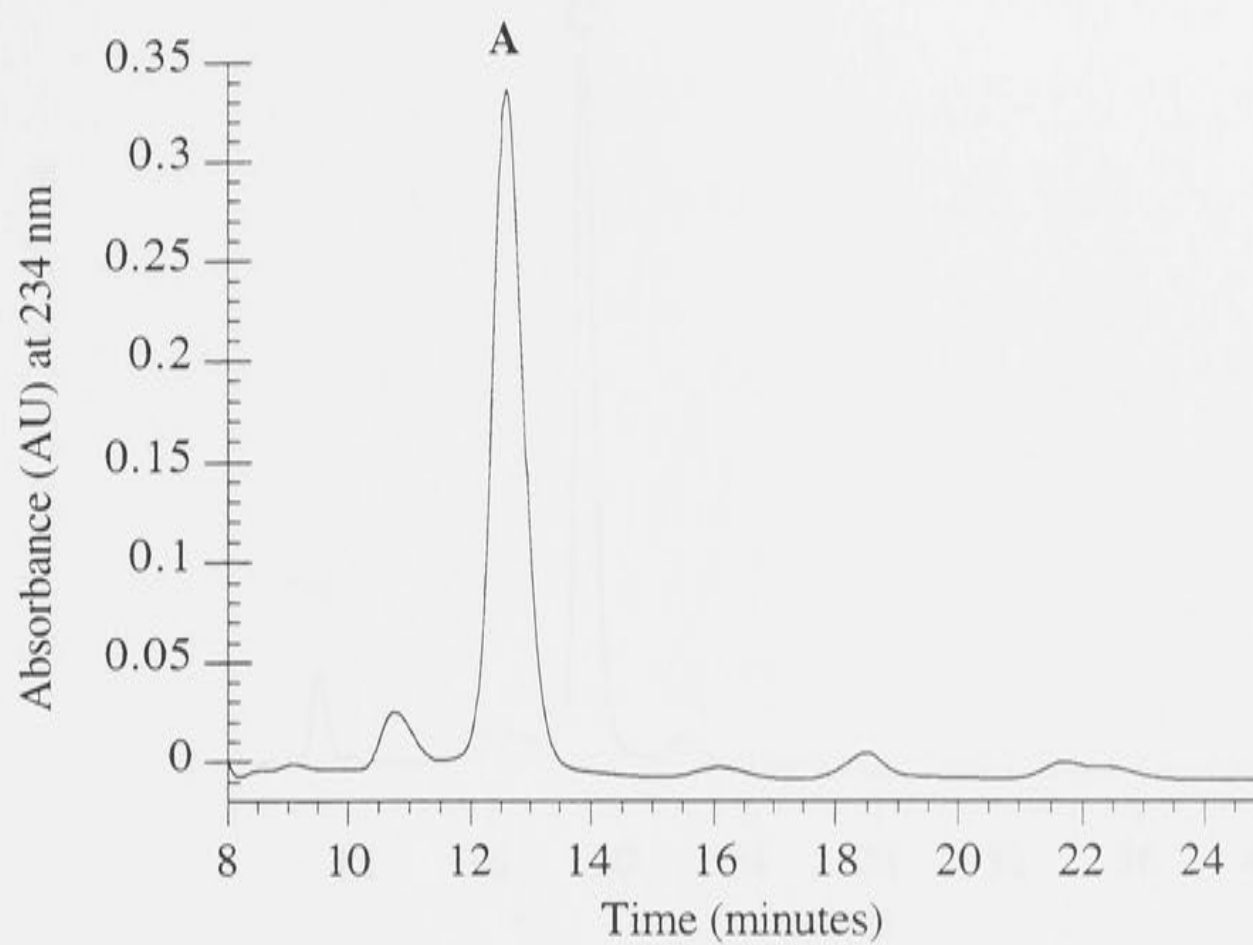
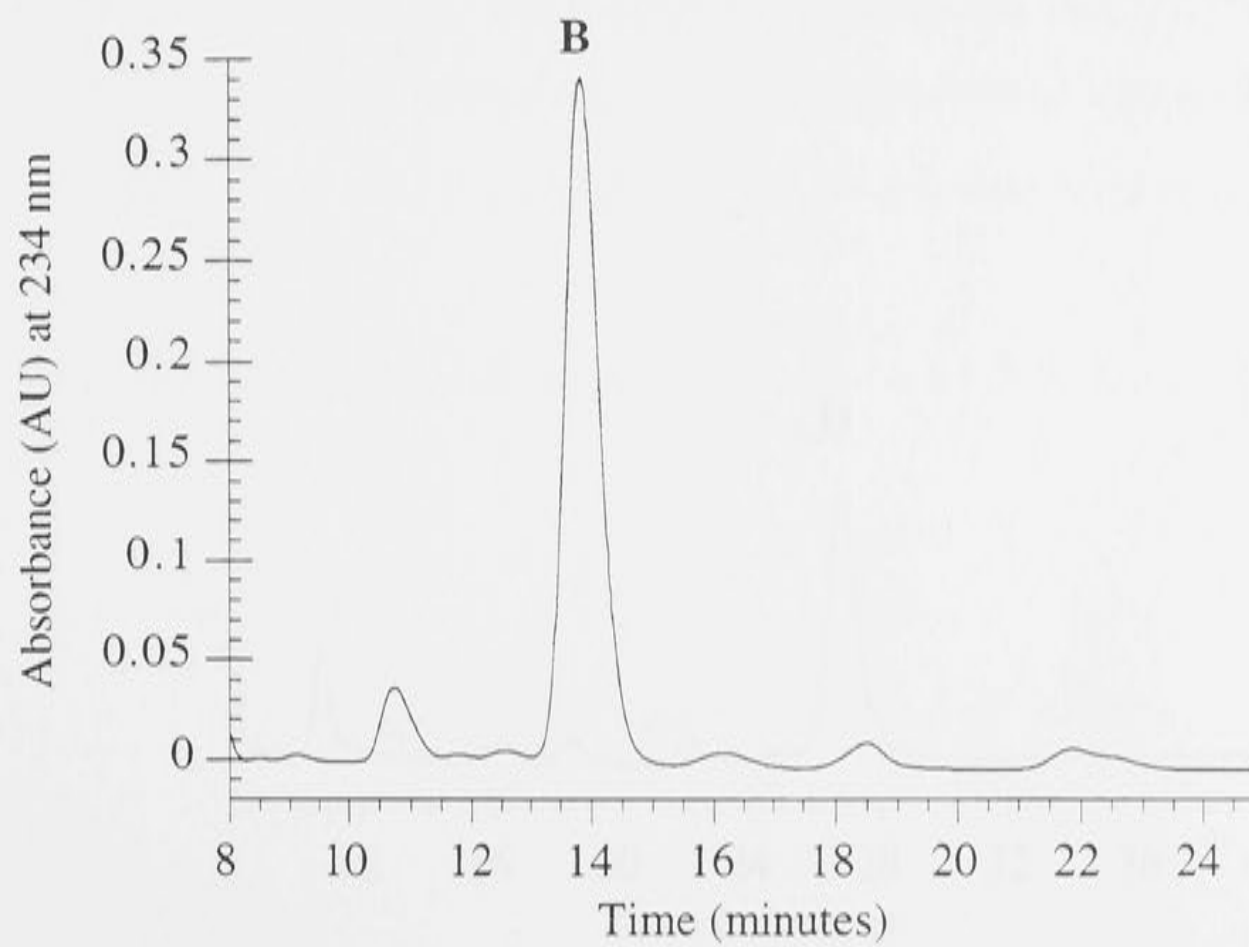
Fig 2.2: HPLC trace for 12-HETE **52** (A)Fig 2.3: HPLC trace for 15-HETE **53** (B)

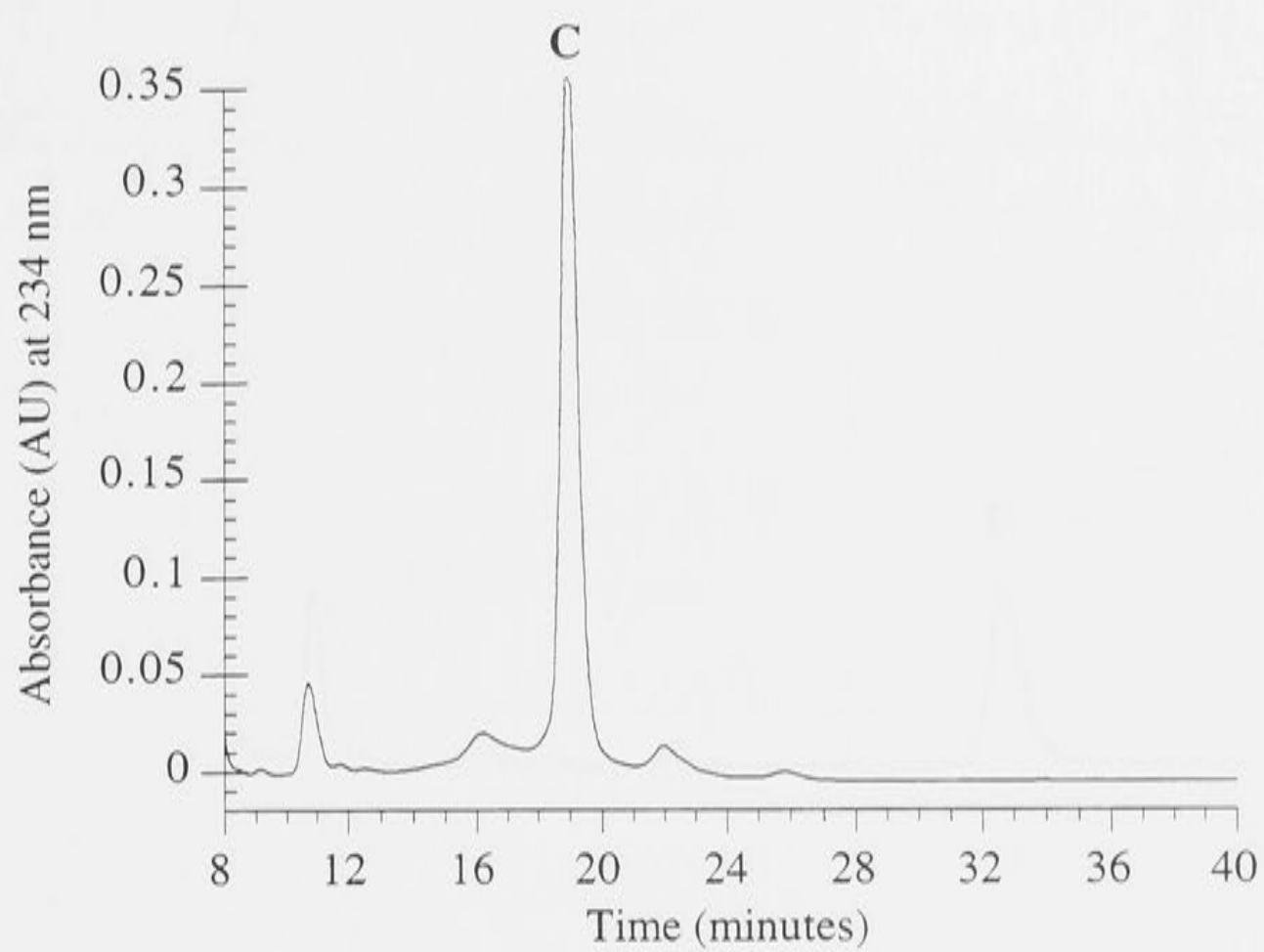
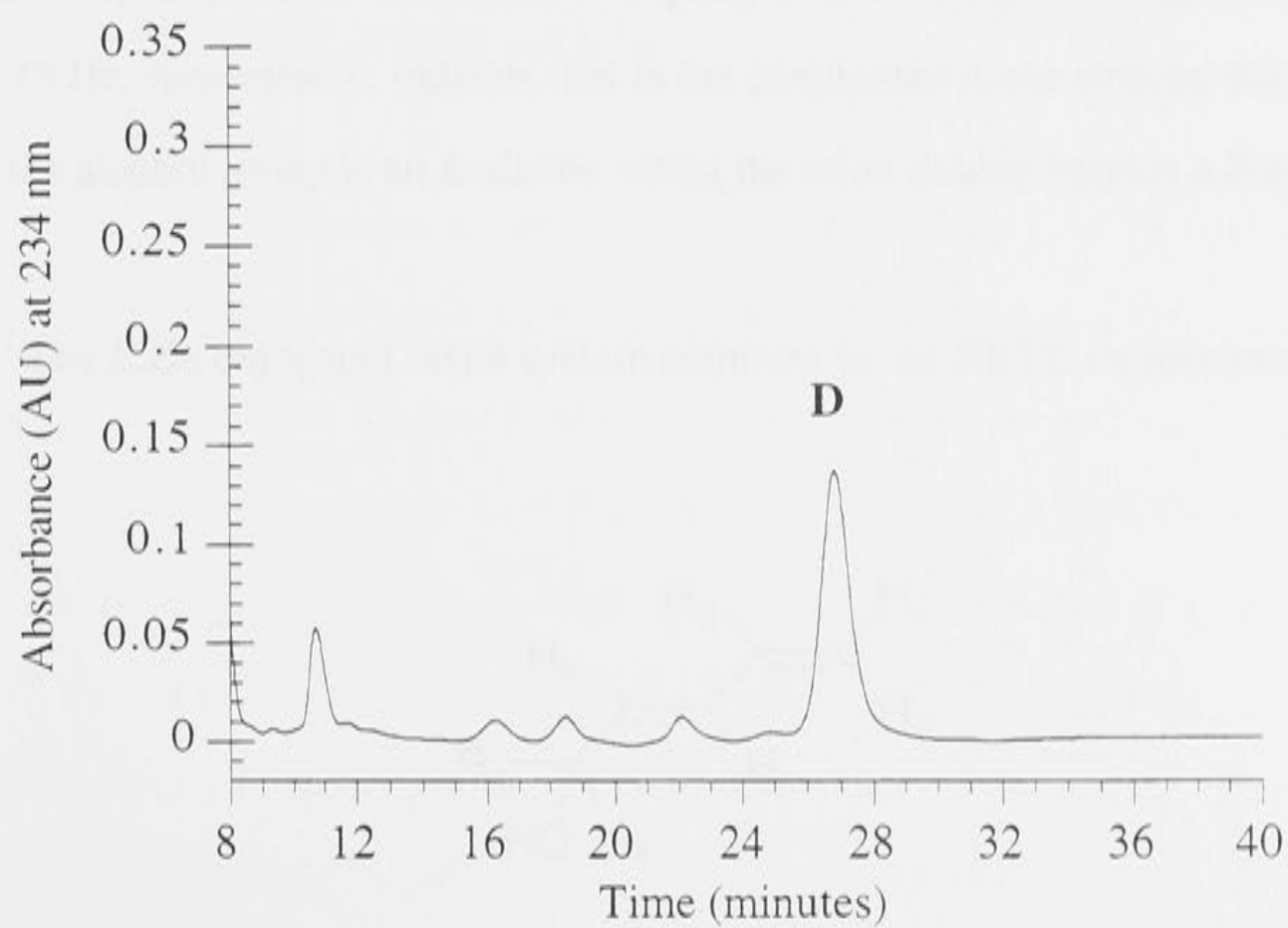
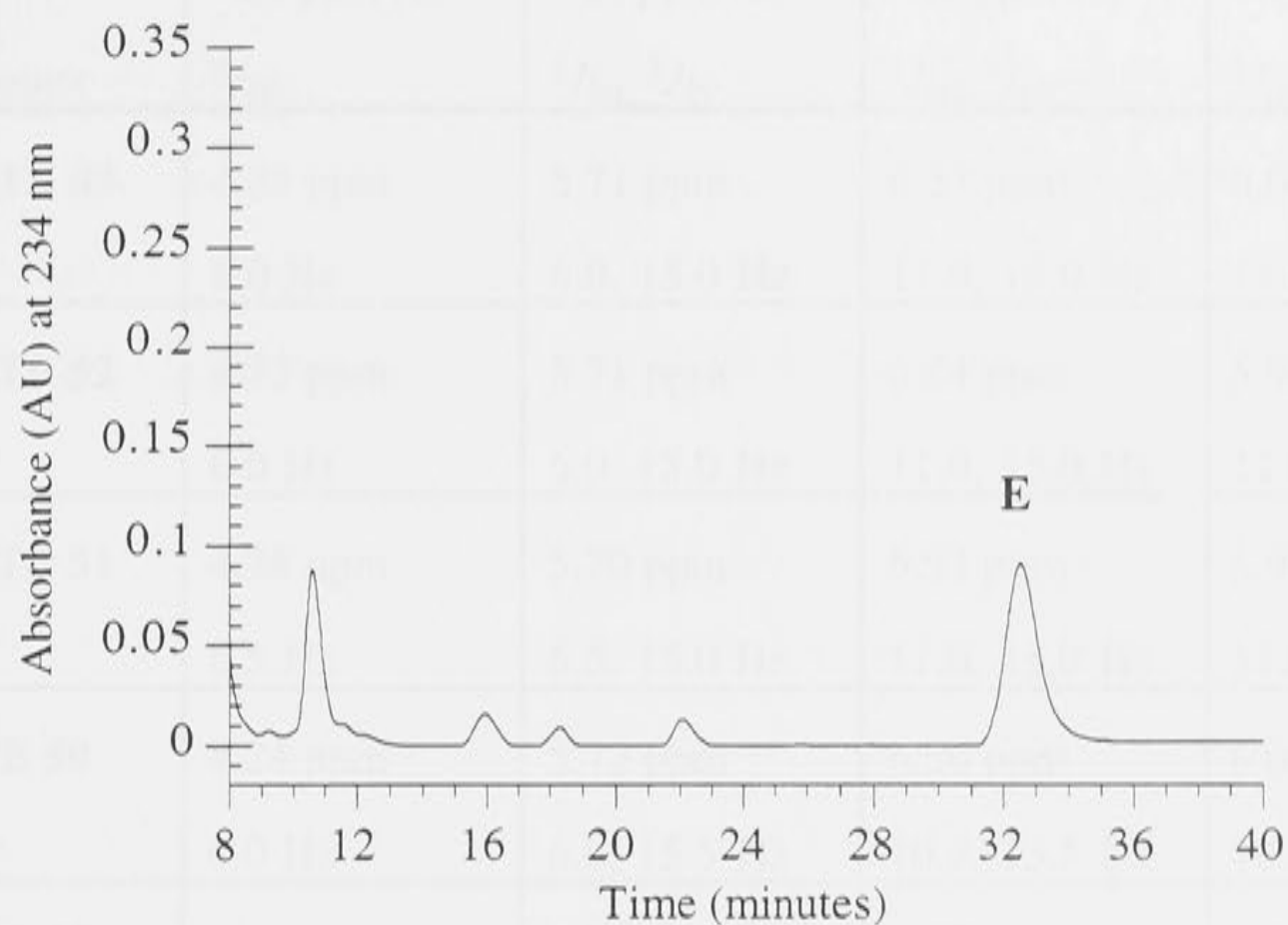
Fig 2.4: HPLC trace for 11-HETE **51** (C)Fig 2.5: HPLC trace for 9-HETE **50** (D)

Fig 2.6: HPLC trace for 8-HETE **49** (**E**)

of the conjugated *E,Z*-diene system, shown in Fig 2.7. As shown in Table 2.5, the chemical shift values and coupling constants for each compound confirmed that each was an HETE isomer but the lack of unique values for each isomer made the assignment of regiochemistry impossible. The vicinal coupling constants $^3J_{bc}$ and $^3J_{de}$ with values of 11 Hz and 15 Hz, respectively, indicate that in the conjugated diene system, the double bond nearest the alcohol group is an *E*-alkene whilst the other double bond is a *Z*-alkene.

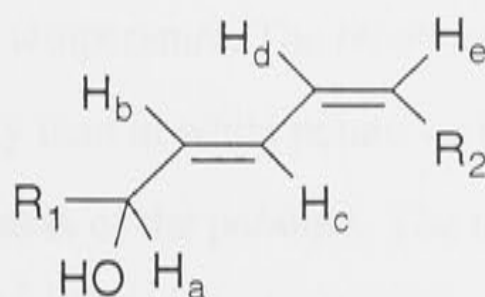
Fig 2.7: The *E,Z*-conjugated diene system common to the HETE regioisomers **48-53**

Table 2.5: Coupling constants and chemical shift values for the HETE regioisomers **49-53** between 4.0 and 7.0 ppm

HETE regioisomer	H _a : ppm (δ) $^3J_{ab}$	H _b : ppm (δ) $^3J_{ba}, ^3J_{bc}$	H _c : ppm (δ) $^3J_{cd}, ^3J_{cb}$	H _d : ppm (δ) $^3J_{dc}, ^3J_{de}$
15-HETE 53	4.23 ppm 6.0 Hz	5.71 ppm 6.0, 15.0 Hz	6.57 ppm 11.0, 15.0 Hz	6.00 ppm 11.0, 11.0 Hz
12-HETE 52	4.33 ppm 6.0 Hz	5.71 ppm 6.0, 15.0 Hz	6.64 ppm 11.0, 15.0 Hz	5.97 ppm 11.0, 11.0 Hz
11-HETE 51	4.25 ppm 6.5 Hz	5.70 ppm 6.5, 15.0 Hz	6.53 ppm 11.0, 15.0 Hz	5.98 ppm 11.0, 11.0 Hz
9-HETE 50	4.24 ppm 6.0 Hz	5.72 ppm 6.0, 15.5 Hz	6.56 ppm 10.5, 15.5 Hz	6.04 ppm 10.5, 10.5 Hz
8-HETE 49	4.24 ppm 6.0 Hz	5.72 ppm 6.0, 15.0 Hz	6.52 ppm 11.0, 15.0 Hz	6.04 ppm 11.0, 11.0 Hz

In summary, following separation of the HETE isomers **49-53** by HPLC, proton nmr spectroscopy was able to confirm the presence of each of the HETE isomers **48-53** but was unable to distinguish between the isomers.

The final HETE isomer to be isolated was 5-HETE **48** using a 5-LO enzyme to oxidise arachidonic acid **3**. Crude potato 5-LO was prepared from potato tubers using a literature procedure by ammonium sulphate fractionation followed by dialysis.⁶¹ The yield of 5-LO activity was much higher if the procedure was performed in a cold room (at 4 °C) than when it was performed at room temperature. The recovered 5-LO activity was found to be greater in the red Pontiac variety than in white potato varieties and was also dependent on seasonal variations in the freshness of the potatoes. The use of nonionic polyoxyethylene detergent (3 mM Brij58) in the extraction buffer also improved the recovered 5-LO activity.⁶⁰ Hence, the highest enzyme activity was recorded for 5-LO prepared from local organically grown red Pontiac potatoes at 4 °C using detergent in the buffer.

(5*S*)-HPETE (*S*)-**13** was prepared from arachidonic acid **3** with crude potato 5(*S*)-LO extract in buffer, derivatised to (5*S*)-HETE (*S*)-**48** with sodium borohydride, extracted into dichloromethane and purified by HPLC.

The composition of mixtures of the HETE isomers **48-53** formed by enzyme catalysed oxidation was compared by HPLC as shown in Figs 2.8 and 2.9. A more polar solvent system (hexane : isopropanol : acetic acid 950 : 50 : 1) than that used earlier was employed to bring the retention time of 5-HETE **48** to less than 15 minutes.

For soybean 15-LO the major peak in Fig 2.8 corresponded to 15-HETE **53** with a retention time of 6.6 minutes. In the literature, the soybean 15-LO is reported to form (15*S*)-HETE (*S*)-**53** as the major HETE product with other regioisomers representing less than 1% of the reaction mixture.³⁵ For potato 5-LO, the predominant peak in Fig 2.9 with a retention time of 13.4 minutes corresponded to 5-HETE **48** and other minor peaks were observed with retention times between 6 and 10 minutes which were not characterised but may be due to other HETE isomers. (5*S*)-HPETE (*S*)-**13** is reported to be the major product of the reaction between arachidonic acid **3** and potato 5-LO with the other minor products being 11-HPETE **12**, 8-HPETE **44** and 9-HPETE **45** in the ratio of 75 : 15 : 5 : 5.⁶¹ Hence, 11-HETE **51**, 8-HETE **49** and 9-HETE **50** would be expected as minor components in the trace shown in Fig 2.9 for the products formed from arachidonic acid **3** using 5-LO. These isomers **49-51** are known to have retention times between those of 15-HETE **53** and 5-HETE **48**.

Following isolation of the six *E,Z*-conjugated diene HETE isomers **48-53** as outlined above, each was analysed by negative ion electrospray tandem mass spectrometry. This technique has not been reported for the analysis of the HETE isomers **48-53** but during the course of this project it was used to analyse a dihydroxylated derivative of arachidonic acid **3**, leukotriene B₄.⁴⁸ In the past three years, tandem mass spectrometry has come into vogue as a method to assign regiochemistry to the HETE isomers **48-53**.^{47,50} Several techniques of sample delivery to the mass spectrometer have been published including

Fig 2.8: HPLC trace for 15-HETE **53** (**B**) formed from arachidonic acid **3** using 15-LO

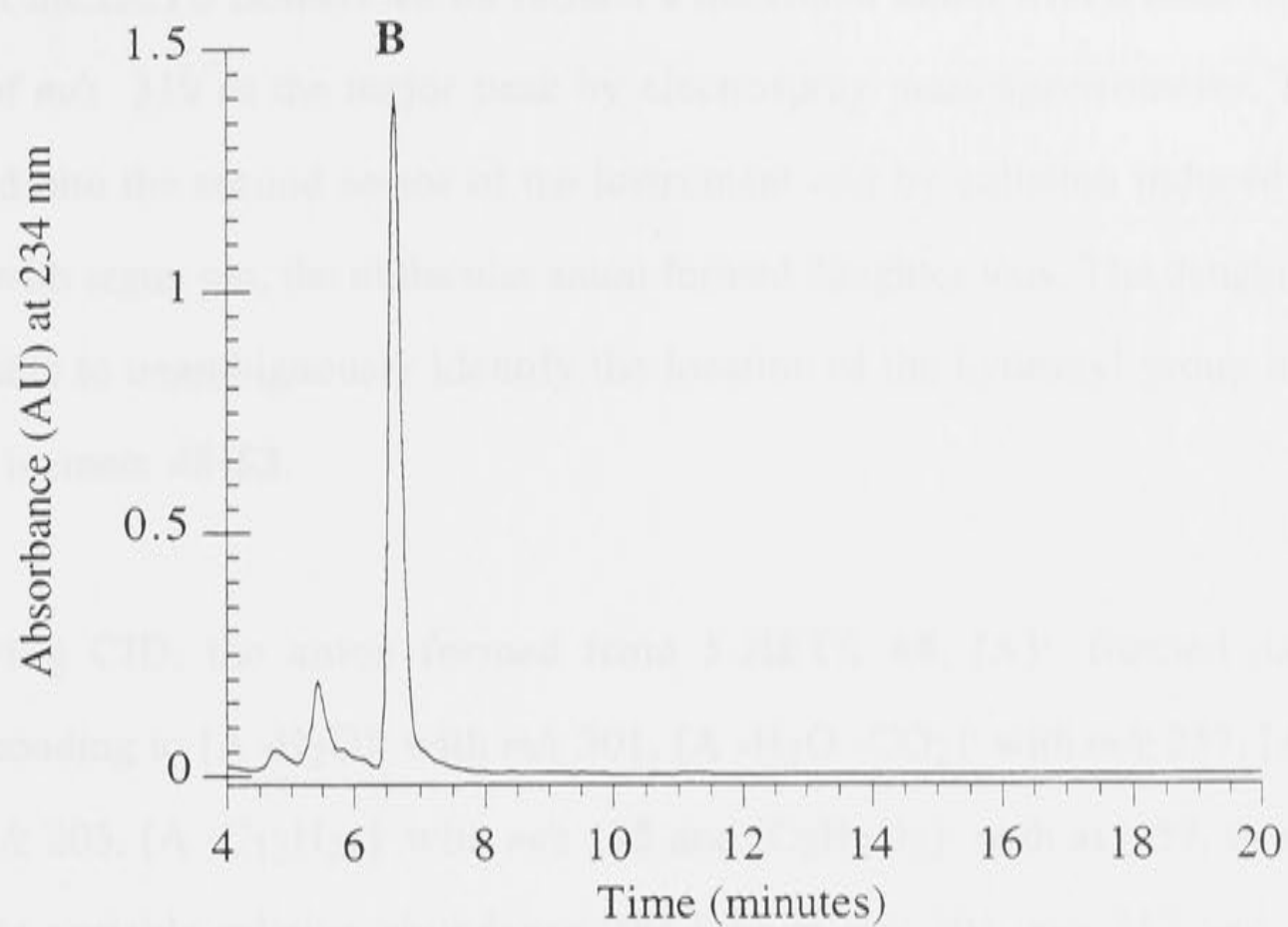
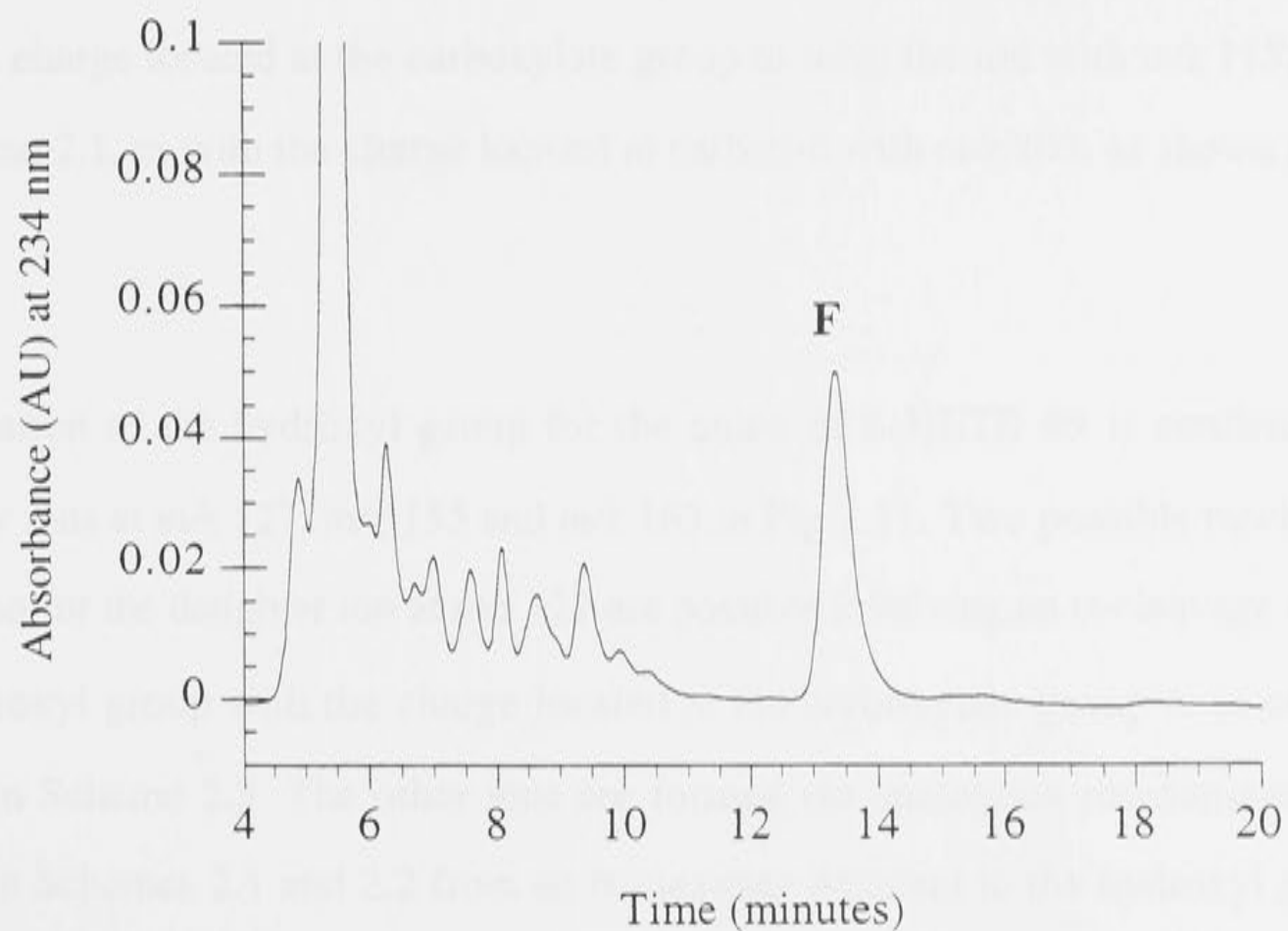


Fig 2.9: HPLC trace for 5-HETE **48** (**F**) formed from arachidonic acid **3** using 5-LO



low energy fast atom bombardment mass spectrometry and liquid secondary ion mass spectrometry.

Each of the HETE isomers **48-53** formed a molecular anion with a mass to charge ratio (m/z) of m/z 319 as the major peak by electrospray mass spectrometry. This ion was directed into the second sector of the instrument and by collision induced dissociation (CID) with argon gas, the molecular anion formed daughter ions. The daughter ions made it possible to unambiguously identify the location of the hydroxyl group in each of the HETE isomers **48-53**.

Following CID, the anion formed from 5-HETE **48**, $[A]^-$, formed daughter ions corresponding to $[A - H_2O]^-$ with m/z 301, $[A - H_2O - CO_2]^-$ with m/z 257, $[A - C_5H_8O_3]^-$ with m/z 203, $[A - C_{15}H_{24}]^-$ with m/z 115 and $[C_2H_3O_2]^-$ with m/z 59, as shown in Fig 2.10. At variable relative abundance, the ions at m/z 301, m/z 257 and m/z 59 were observed in the spectra of all six HETE regioisomers **48-53**. The location of the hydroxyl group for the anion of 5-HETE **48** is confirmed by the daughter ions at m/z 115 and m/z 203. These ions are derived from an α -cleavage adjacent to the hydroxyl group with the charge located at the carboxylate group to form the ion with m/z 115, as shown in Scheme 2.1, or with the charge located at carbon-6 with m/z 203, as shown in Scheme 2.2.

The location of the hydroxyl group for the anion of 8-HETE **49** is confirmed by the daughter ions at m/z 127, m/z 155 and m/z 163 in Fig 2.11. Two possible mechanisms of formation for the daughter ion at m/z 127 are possible involving an α -cleavage adjacent to the hydroxyl group with the charge located at the carboxylate group or at carbon-7 as shown in Scheme 2.3. The other ions are formed *via* analogous mechanisms to those shown in Schemes 2.1 and 2.2 from an α -cleavage adjacent to the hydroxyl group with the charge located at the carboxylate group for the ion at m/z 155 or at carbon-9 for the ion at m/z 163.

Fig 2.10: The electrospray MS/MS spectrum (5*S*)-HETE (*S*)-48

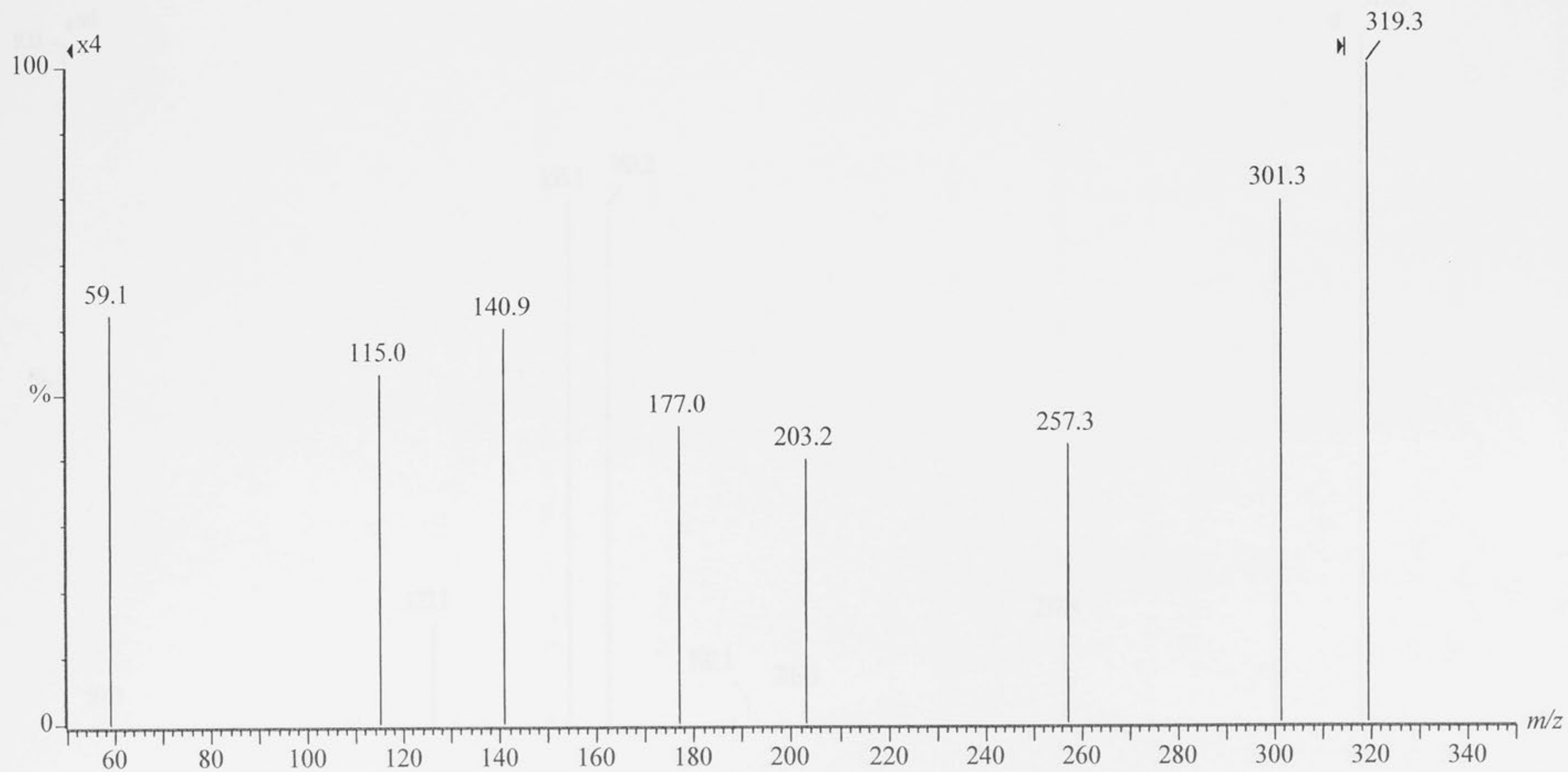
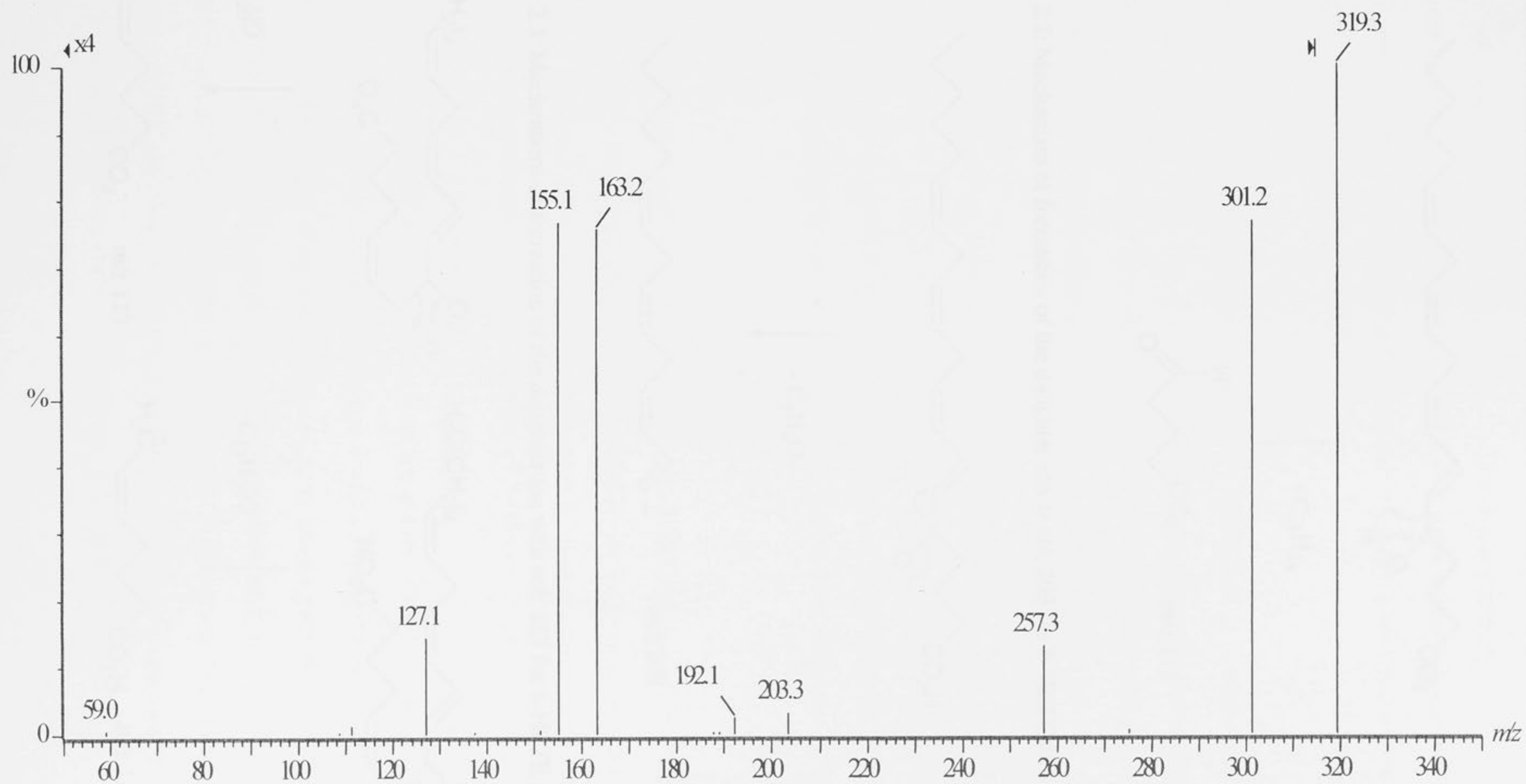


Fig 2.11: The electrospray MS/MS spectrum for 8-HETE **49**



The location of the hydroxyl group for the anion of 9-HETE **50** is confirmed by the daughter ions at m/z 151 and m/z 167 in Fig 2.12. The ions are formed *via* analogous mechanisms to those shown in Schemes 2.1 and 2.2 from an α -cleavage adjacent to the hydroxyl group with the charge located at the carboxylate group for the ion at m/z 167 or at carbon-10 for the ion at m/z 151.

The location of the hydroxyl group for the anion of 11-HETE **51** is confirmed by the daughter ion at m/z 167 in Fig 2.13. The ion is formed *via* an analogous mechanism to that shown in Scheme 2.3 from an α -cleavage adjacent to the hydroxyl group with the charge located either at the carboxylate group or at carbon-10.

The location of the hydroxyl group for the anion of 12-HETE **52** is confirmed by the daughter ion at m/z 179 in Fig 2.14. This ion is formed *via* an analogous mechanism to that shown in Scheme 2.3 from an α -cleavage adjacent to the hydroxyl group with the charge located at the carboxylate group for the ion at m/z 179 or at carbon-11 for the ion at m/z 179.

The location of the hydroxyl group for the anion of 15-HETE **53** is confirmed by the daughter ion at m/z 219 in Fig 2.15. This ion is formed *via* an analogous mechanism to that shown in Scheme 2.3 from an α -cleavage adjacent to the hydroxyl group with the charge located at the carboxylate group or at carbon-14 for the ion at m/z 151.

The structures of the HETE isomers **48-53** can be represented by the general structure shown in Scheme 2.4, in which the alkyl groups R and R' differ for each isomer. As summarised in Table 2.6 and illustrated in Scheme 2.4, the anions formed from 15-HETE **53**, 12-HETE **52**, 11-HETE **51** and 8-HETE **49** form daughter ions of the general type $[\text{HR}'\text{CO}_2]^-$ whereas 9-HETE **50**, 8-HETE **49** and 5-HETE **48** formed daughter ions of the general type $[\text{R}]^-$ and $[\text{O}=\text{CHR}'\text{CO}_2]^-$. Each of the three types of characteristic ion is formed by cleavage of a carbon-carbon bond adjacent to the hydroxyl group.

Fig 2.12: The electrospray MS/MS spectrum for 9-HETE **50**

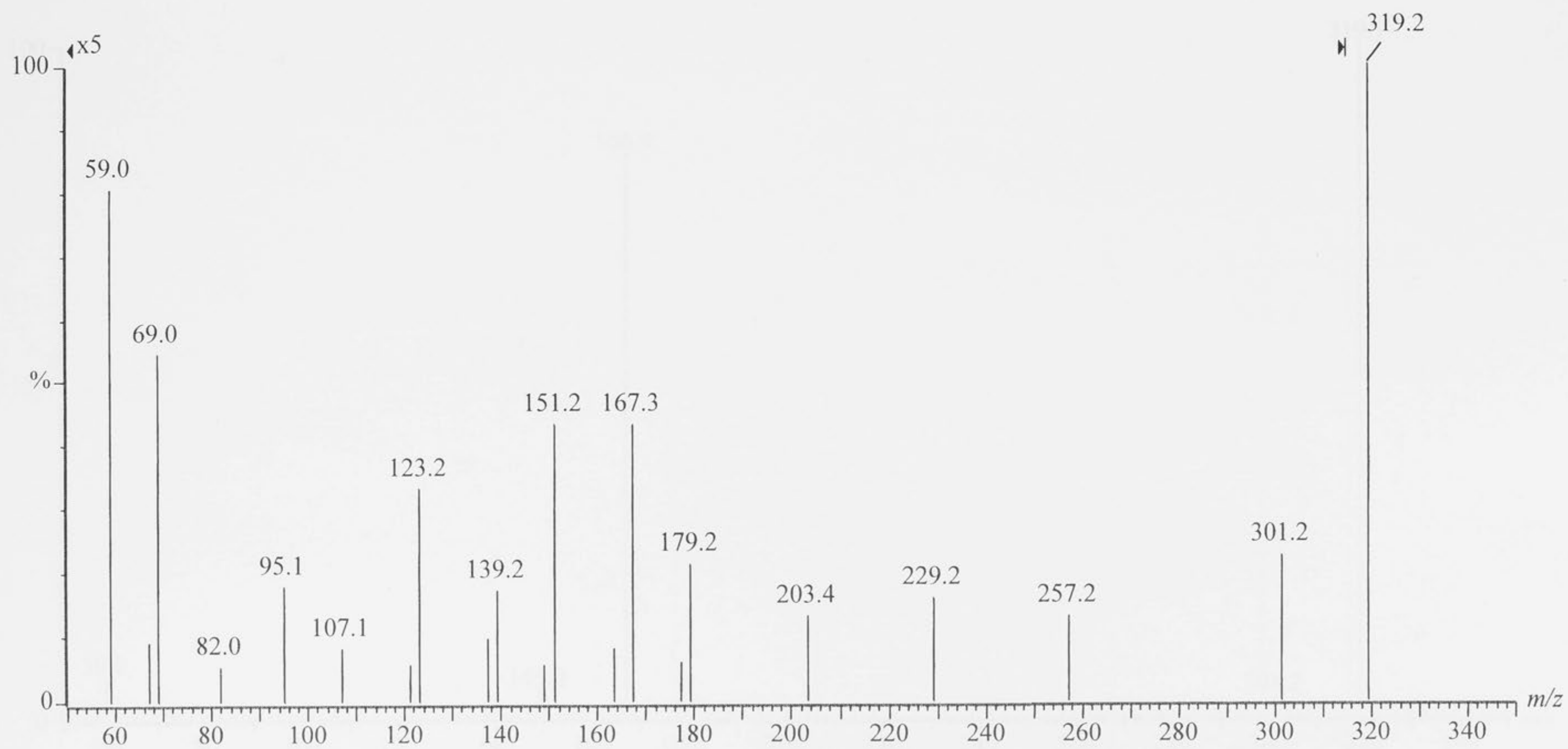


Fig 2.13: The MS/MS spectrum for 11-HETE **51**

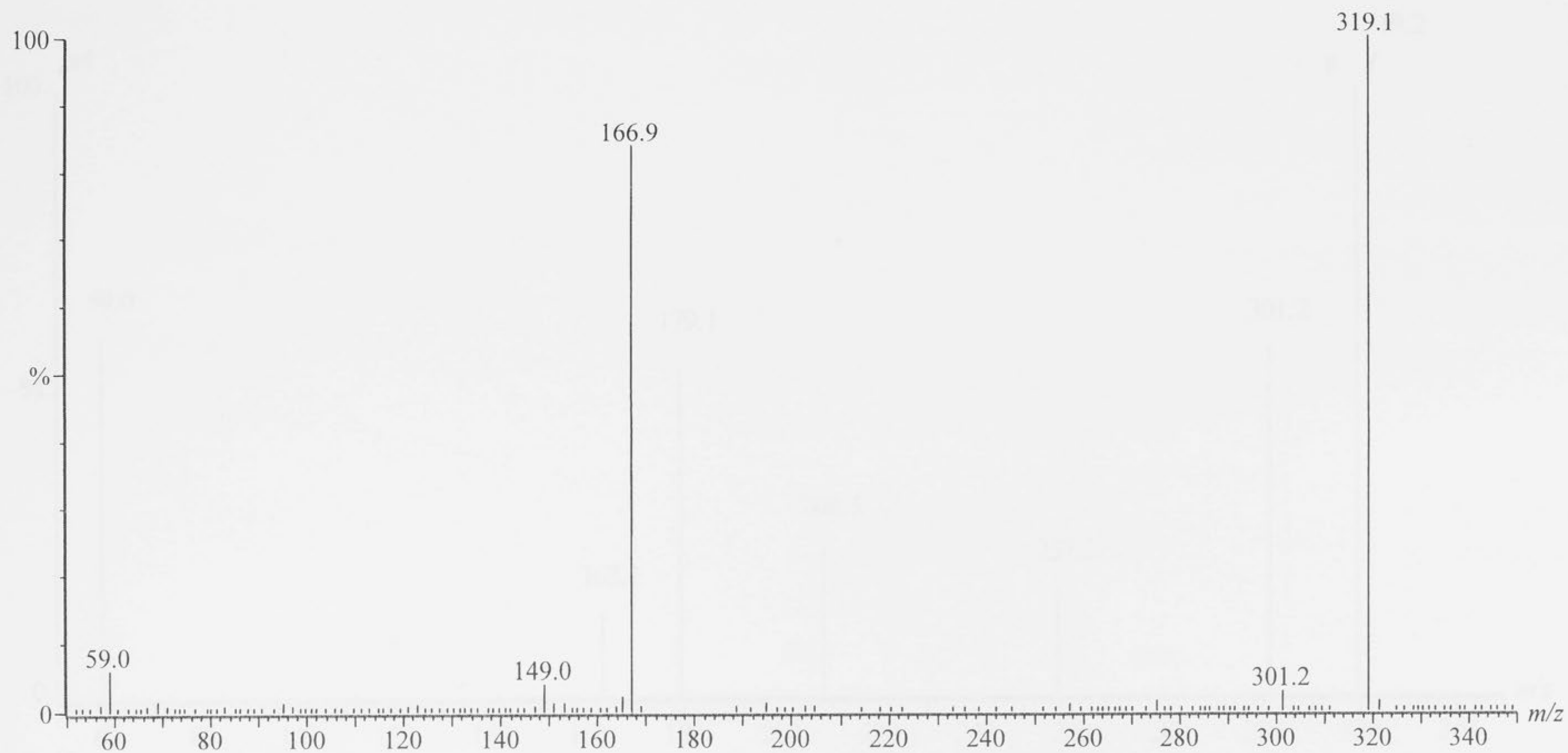


Fig 2.14: The electrospray MS/MS spectrum for 12-HETE **52**

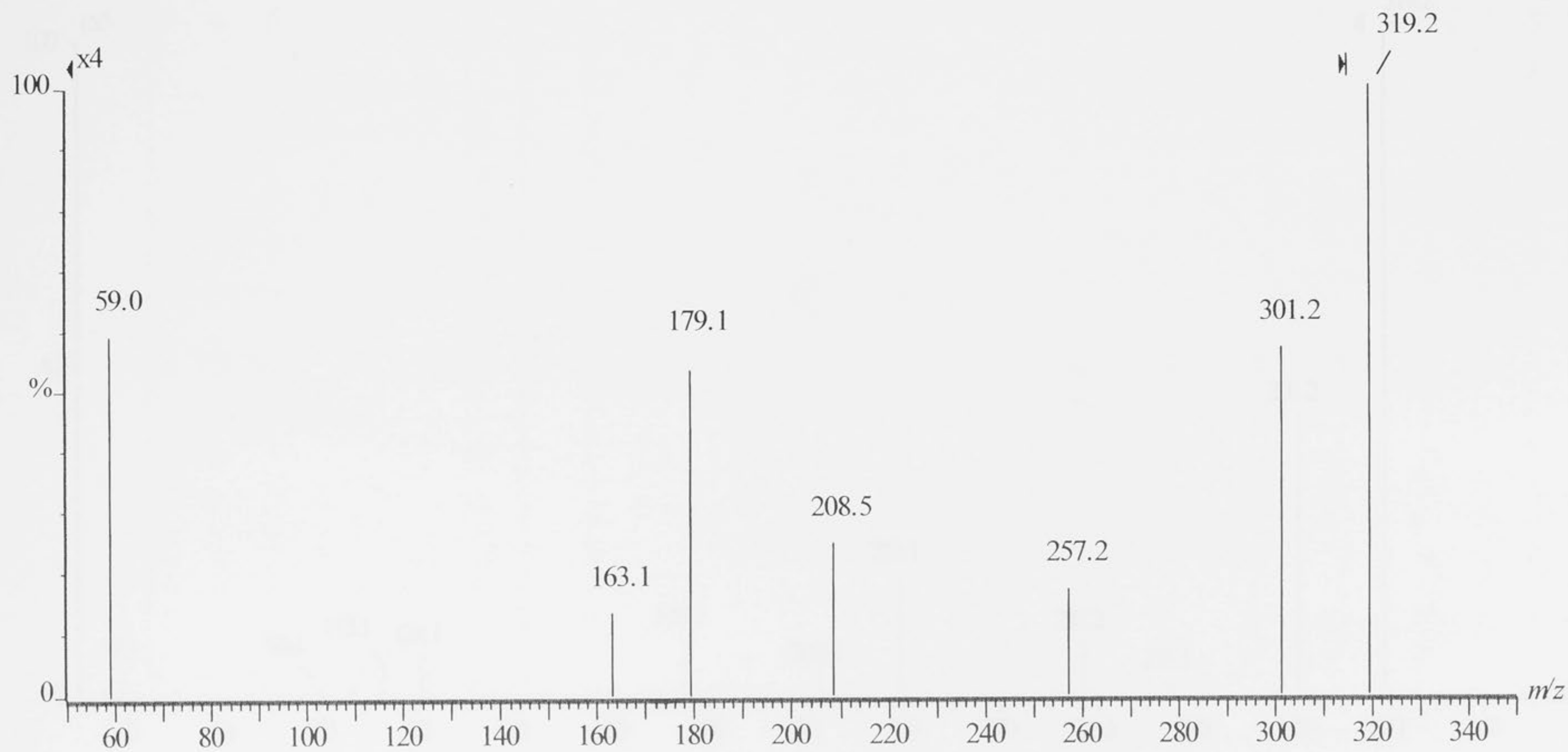
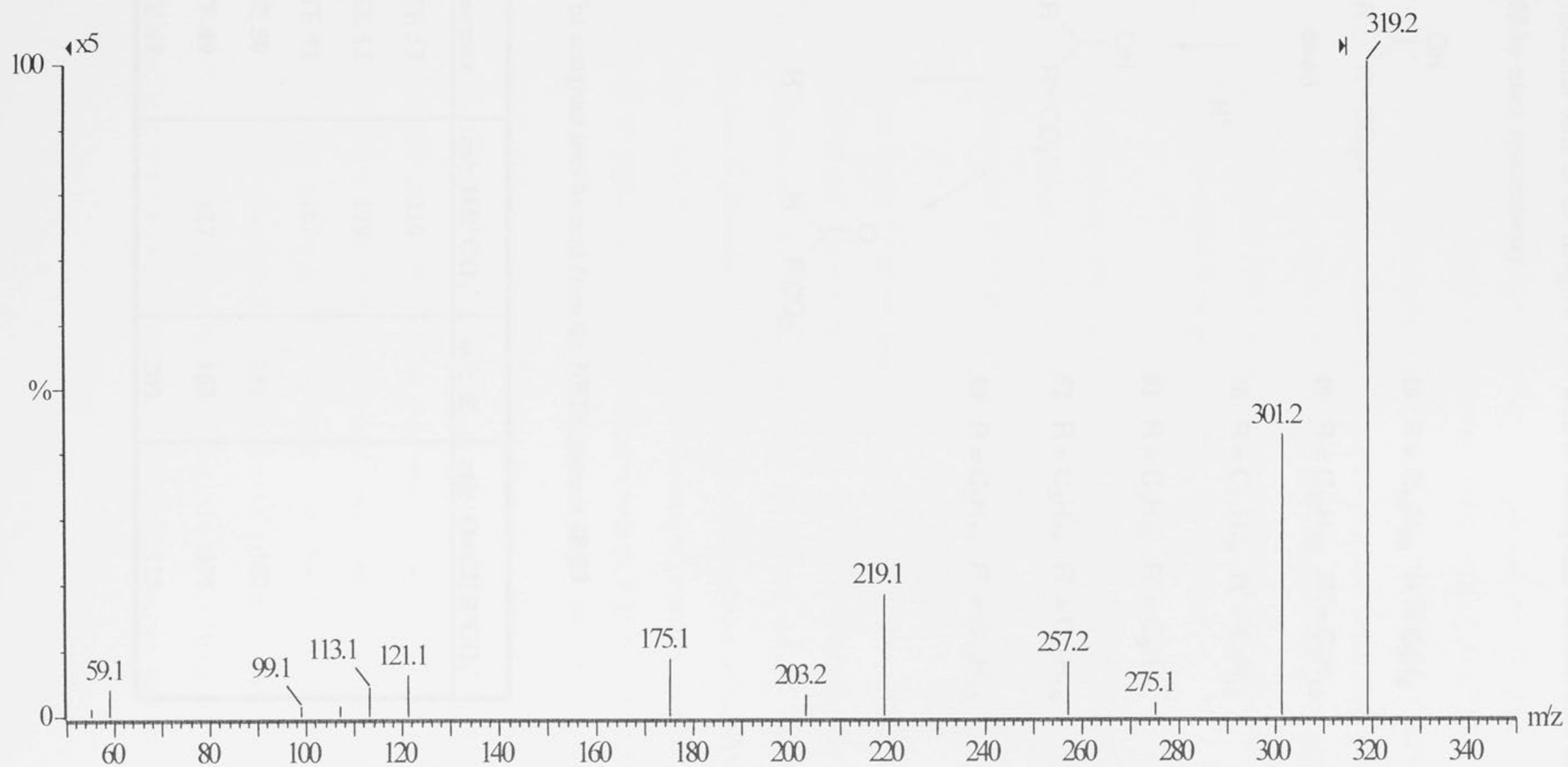


Fig 2.15: The electrospray MS/MS spectrum for 15-HETE **53**



Scheme 2.4: General form of the daughter ions used to distinguish between the HETE isomers **48-53** by mass spectrometry

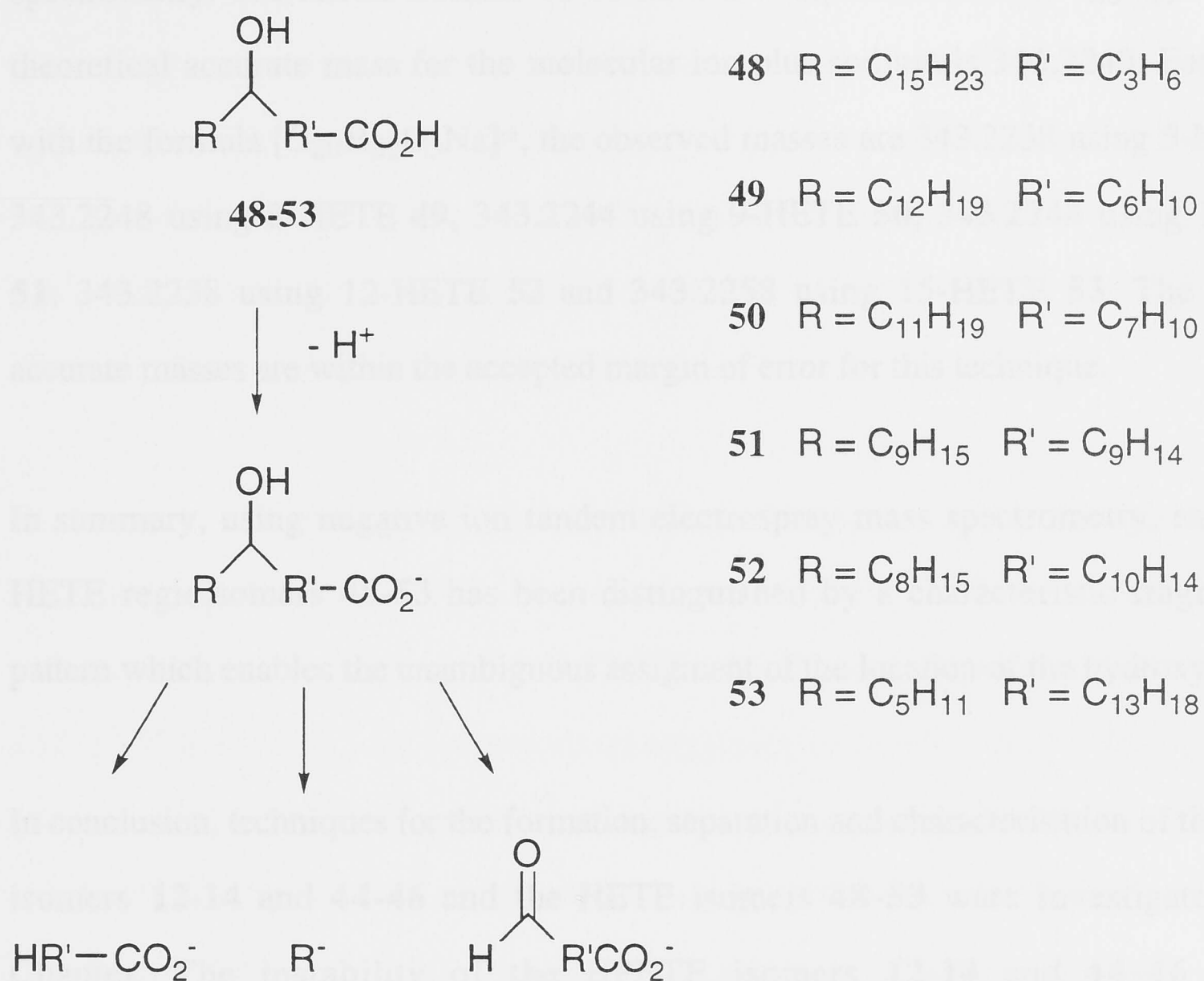


Table 2.6: The daughter ions formed from the HETE isomers **48-53**

HETE isomer	m/z $\text{HR}'\text{CO}_2^-$	m/z R^-	m/z $\text{O}=\text{CHR}'\text{CO}_2^-$
15-HETE 53	219	-	-
12-HETE 52	179	-	-
11-HETE 51	167	-	-
9-HETE 50	-	151	167
8-HETE 49	127	163	155
5-HETE 48	-	203	115

For each of the HETE isomers **48-53**, the accurate mass of the molecular ion plus sodium was recorded by high resolution positive ion tandem electrospray mass spectrometry. The HETE isomers **48-53** have the empirical formula $C_{20}H_{32}O_3$ and the theoretical accurate mass for the molecular ion plus sodium is 343.2249. For the ions with the formula $[C_{20}H_{32}O_3Na]^+$, the observed masses are 343.2238 using 5-HETE **48**, 343.2248 using 8-HETE **49**, 343.2244 using 9-HETE **50**, 343.2244 using 11-HETE **51**, 343.2238 using 12-HETE **52** and 343.2258 using 15-HETE **53**. The recorded accurate masses are within the accepted margin of error for this technique.

In summary, using negative ion tandem electrospray mass spectrometry, each of the HETE regioisomers **48-53** has been distinguished by a characteristic fragmentation pattern which enables the unambiguous assignment of the location of the hydroxyl group.

In conclusion, techniques for the formation, separation and characterisation of the HPETE isomers **12-14** and **44-46** and the HETE isomers **48-53** were investigated in this Chapter. The instability of the HPETE isomers **12-14** and **44-46** towards chromatography has been confirmed with (15*S*)-HPETE (*S*)-**14** and an HPLC system for the detection of the HETE isomers **48-53** has been developed, with the use of (15*S*)-HPETE (*S*)-**14** and (15*S*)-HETE (*S*)-**53** derived from arachidonic acid **3** using soybean 15-LO. The prooxidant nature of α -tocopherol **15** in the formation of the HPETE isomers **12-14** and **44-46** using thin film autoxidation has been monitored by HPLC and optimum conditions for the formation of the six *E,Z*-conjugated diene HETE isomers **48-53** were found. The HETE isomers **48-53** have been separated and isolated by HPLC and the isomers **49-53** were analysed by proton nmr spectroscopy but this method confirmed that each compound was a conjugated *E,Z*-diene regioisomer without distinguishing between the isomers **49-53**. Using negative ion tandem electrospray mass spectrometry a method for the assignment of regiochemistry to the HETE isomers **48-53** has been developed based on the unique daughter ions formed by the molecular anion of each regioisomer.

An HPLC study of the oxidation of β -oxa and β - and γ -thia fatty acid derivatives

As discussed in the Introduction, synthetic analogues of PUFAs are of interest as potential pharmaceuticals and in experiments to examine enzyme mechanism and substrate specificity. In humans, LO inhibitors are used to treat conditions involving inflammation such as asthma and rheumatoid arthritis. LO substrates and products, including synthetic analogues of naturally occurring PUFAs, regulate LO activity by mechanisms such as feedback inhibition and competitive inhibition.

Within our research group the synthetic analogues of straight chain PUFAs, [6'Z,9'Z,12'Z]-octadecatrienyloxyacetic acid **83**, [9'Z,12'Z,15'Z]-octadecatrienyloxyacetic acid **84**, [5'Z,8'Z,11'Z,14'Z]-eicosatetraenylthioacetic acid **85** and [5'Z,8'Z,11'Z,14'Z]-eicosatetraenylthiopropionic acid **86** have been prepared.^{108,109} It has been shown that these compounds **83-86** are not suitable substrates for metabolism *via* β -oxidation, the main route of PUFA breakdown in vertebrates (Scheme 1.1). Consequently, the half lives of such compounds *in vivo* are proposed to be longer and the biological activity associated with these synthetic compounds **83-86** will also be sustained compared to unmodified PUFAs. The remaining synthetic compounds **87-89** introduced earlier were not studied in the work described in this Chapter because they had not been prepared in sufficient quantities when this work was undertaken. However, the results presented in this Chapter led to the comparison of the compounds **83-89** in the research presented in Chapters 3 and 4.

The aim of the research presented in this Chapter was to investigate the autoxidation of the synthetic compounds **83-86** and their interactions with 5-LO and 15-LO, as well as their effect on the autoxidation of arachidonic acid **3** and the interaction of that compound with 5-LO and 15-LO. Enzyme mediated oxidation and thin film autoxidation are techniques which could potentially be applied to prepare oxidation products from the compounds **83-86**.

Autoxidation can be used to compare the chemical stability of the synthetic compounds **83-86** with that of arachidonic acid **3**. By following an analogous method to that used in Chapter 1 to prepare the HETE isomers **48-53** from arachidonic acid **3**, it was expected that thin film autoxidation could be used to prepare regioisomers of the principal oxidation products from each of the synthetic compounds **83-86**.

As the synthetic compounds **83-86** are analogues of natural PUFAs and therefore possibly LO substrates, they have the potential to competitively inhibit the consumption of arachidonic acid **3**, for example, through competitive binding to the enzyme without reacting or by binding and undergoing oxidation. Products of LO catalysed oxidation of the synthetic PUFAs **83-86** will potentially regulate LO activity by feedback inhibition.

A reversed phase HPLC assay was developed to simultaneously measure relative amounts of arachidonic acid **3** and any one of the synthetic compounds **83-86** in the presence of an internal standard, the saturated compound lauric acid [12:0]. Lauric acid cannot rapidly undergo autoxidation or LO catalysed oxidation so it is considered unreactive in the systems studied. Using octadecylsilane (ODS) derivatised silica and a solvent system containing acetonitrile and 30 mM phosphoric acid (80 : 20), the retention times of the compounds studied were: lauric acid, 8.0 minutes; the (n-6)- β -oxa compound **83**, 12.7 minutes; the (n-3)- β -oxa compound **84**, 12.8 minutes; arachidonic acid **3**, 13.7 minutes; the β -thia compound **85**, 18.6 minutes; and the γ -thia compound **86**, 20.5 minutes.

The HPLC assay was first used to determine whether each of the synthetic compounds **83-86** underwent autoxidation under an atmosphere of oxygen at a similar rate to arachidonic acid **3**. A measure of the chemical stability of the synthetic compound **83** is illustrated in Figs 3.1 and 3.2, in which a 1 : 1 mixture of arachidonic acid **3** and the β -oxa-compound **83** was analysed by HPLC before and after 7 days of thin film autoxidation.

Fig 3.1: HPLC analysis of the mixture containing lauric acid (**A**), the (n-6)- β -oxa compound **83** (**B**) and arachidonic acid **3** (**C**) prior to thin film autoxidation

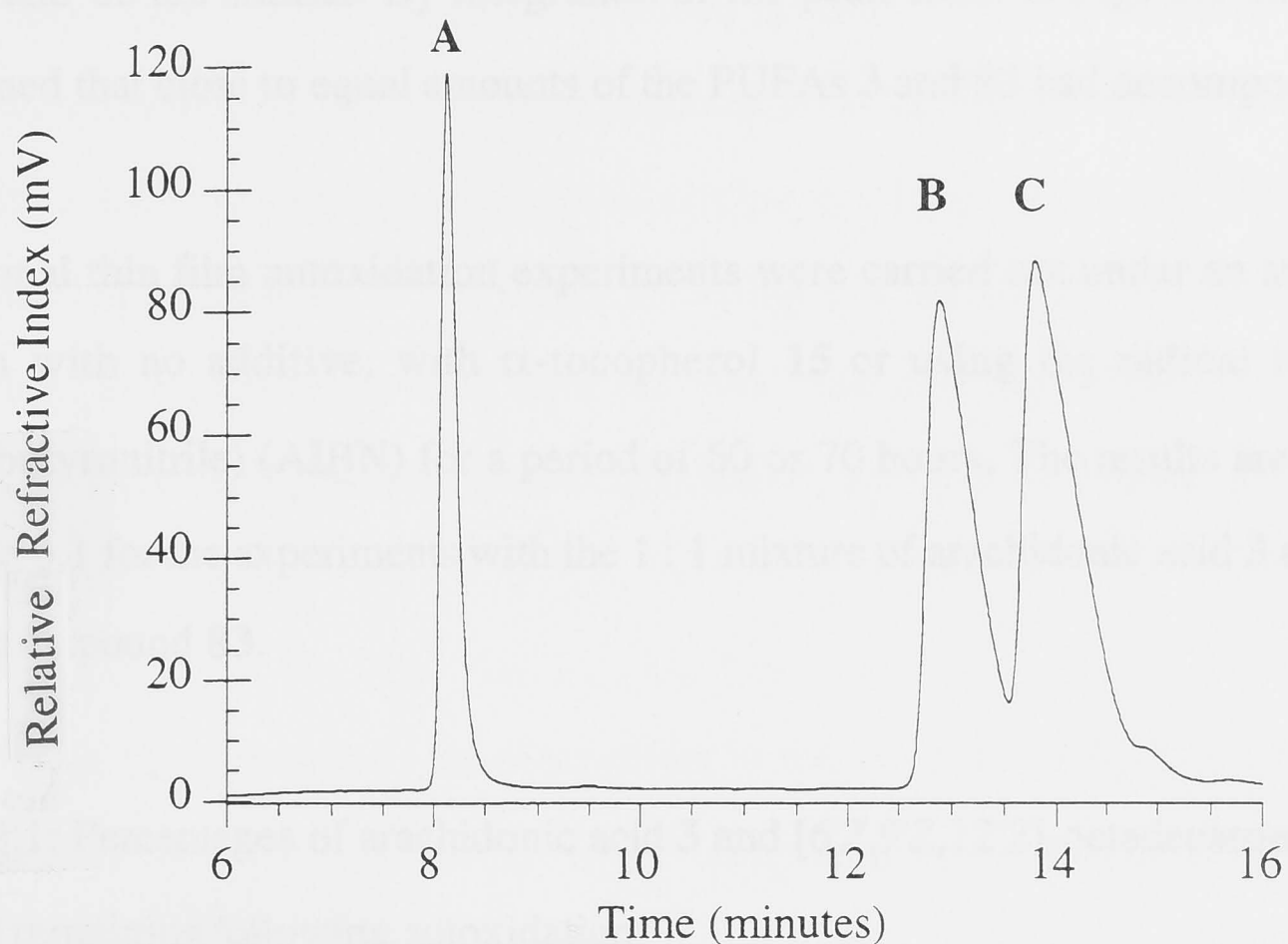
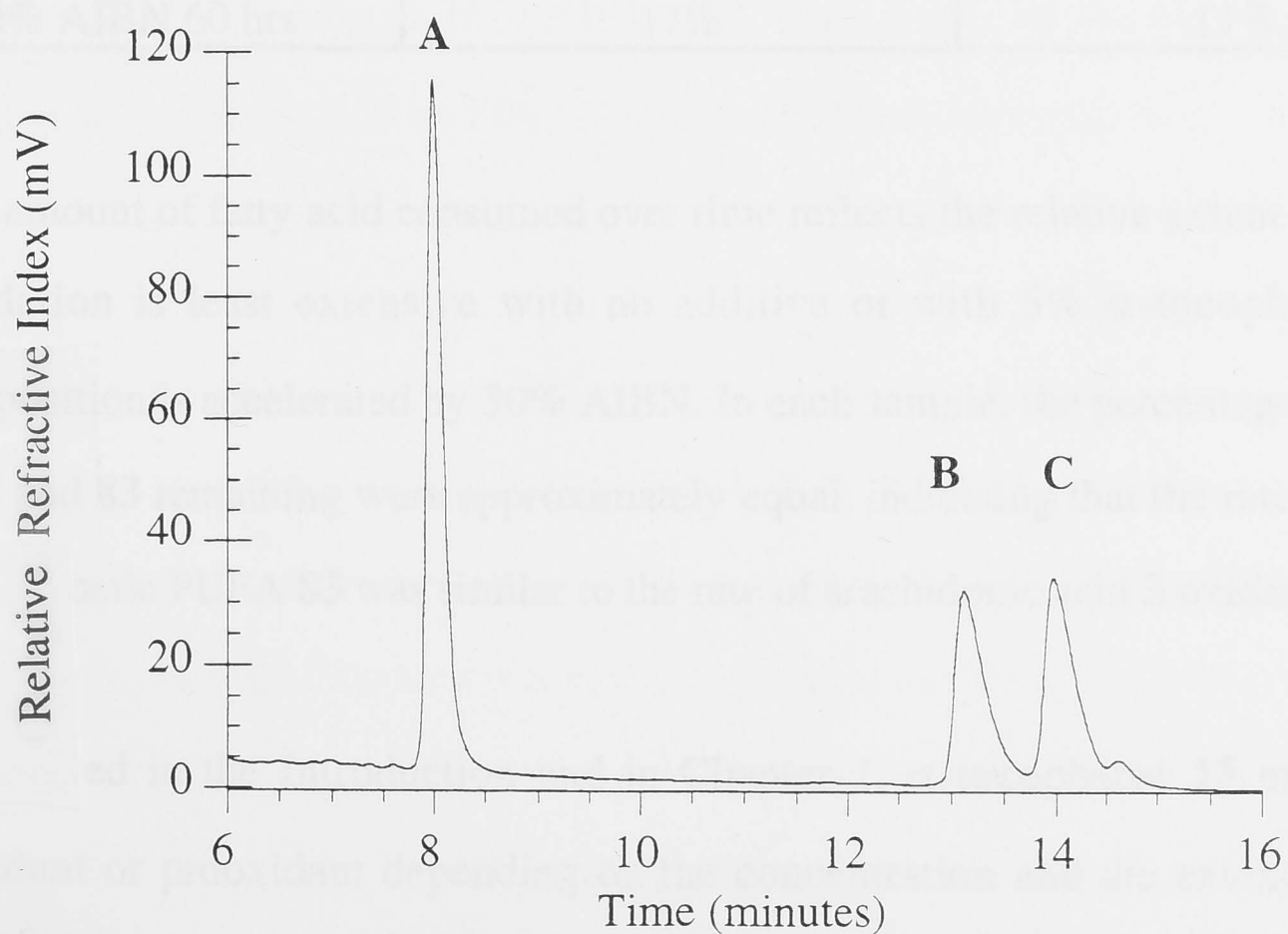


Fig 3.2: HPLC analysis of the mixture containing lauric acid (**A**), the (n-6)- β -oxa compound **83** (**B**) and arachidonic acid **3** (**C**) following 7 days of autoxidation



In viewing the two HPLC traces above it is clear that the peaks for the two polyunsaturated compounds **83** and **3** are each reduced in area by more than half after a week of autoxidation and the relative amounts of the two PUFAs **83** and **3** remain close to equal. Hence, the rates of autoxidation of arachidonic acid **3** and the synthetic compound **83** are similar. By integration of the peak areas in Figs 3.1 and 3.2, it was confirmed that close to equal amounts of the PUFAs **3** and **83** had decomposed.

Additional thin film autoxidation experiments were carried out under an atmosphere of oxygen with no additive, with α -tocopherol **15** or using the radical initiator azo-bis(isobutyronitrile) (AIBN) for a period of 60 or 70 hours. The results are summarised in Table 3.1 for the experiments with the 1 : 1 mixture of arachidonic acid **3** and the (n-6)- β -oxa compound **83**.

Table 3.1: Percentages of arachidonic acid **3** and [6'Z,9'Z,12'Z]-octadecatrienyloxyacetic acid **83** remaining following autoxidation

Autoxidation method	20:4(n-6) 3	21:3(n-6)- β -oxa 83
no additive 70 hours	97%	88%
no additive 7 days	23%	27%
5% α -tocopherol 15 60 hrs	93%	95%
30% AIBN 60 hrs	17%	11%

As the amount of fatty acid consumed over time reflects the relative extent of oxidation, autoxidation is least extensive with no additive or with 5% α -tocopherol **15** and decomposition is accelerated by 30% AIBN. In each sample, the percentages of the fatty acids **3** and **83** remaining were approximately equal, indicating that the rate of oxidation of the synthetic PUFA **83** was similar to the rate of arachidonic acid **3** oxidation.

As discussed in the Introduction and in Chapter 1, α -tocopherol **15** may act as an antioxidant or prooxidant depending on the concentration and the extent of reaction.

Under the conditions used in this experiment, α -tocopherol **15** has no significant effect on the oxidation of the PUFAs **3** and **83**.

After 70 hours of autoxidation with no additive, less than 15% of each of the PUFAs **3** and **83** decomposed. Decomposition increased by more than fivefold following a further 4 days of autoxidation. The oxidation products build up relatively slowly over the first three days and the decomposition of unstable products of oxidation increases the concentration of radicals in the mixture. During a week of autoxidation, the radicals accelerate the oxidation process over the final four days by taking part in hydrogen transfer reactions and produce pentadienyl radicals which form oxidation products from the fatty acids **3** and **83**.

In summary, the presence of the ether group in the synthetic (n-6)- β -oxa compound **83** does not alter the chemical stability of the compound **84** relative to arachidonic acid **3** because each compound undergoes autoxidation at a similar rate, under a variety of conditions.

By analogy with the four autoxidation experiments outlined in Table 3.1, a 1 : 1 mixture of the synthetic (n-3)- β -oxa compound **84** and arachidonic acid **3** was allowed to decompose by thin film autoxidation for various periods in the absence of additive, with 5% α -tocopherol **15** or with 10% AIBN. The percentages of the fatty acids **3** and **84** remaining were determined by HPLC and the conditions and results are summarised in Table 3.2.

In Table 3.2, the amounts of the two compounds **3** and **84** remaining in each case are close to equal so the rates of oxidation of each compound are similar. 5% α -tocopherol **15** did not markedly alter the rate of PUFA oxidation whereas 10% AIBN accelerated PUFA decomposition. During a week of autoxidation the build up of unstable oxidation products over the first three days of autoxidation with no additive accelerated the rate of

Table 3.2: Percentages of arachidonic acid **3** and [9'Z,12'Z,15'Z]-octadecatrienyloxyacetic acid **84** recovered following autoxidation

Autoxidation method	20:4(n-6) 3	21:3(n-3)- β -oxa 84
no additive 70 hours	92%	102%
no additive 7 days	30%	41%
5% α -tocopherol 15 60 hrs	90%	90%
10% AIBN 60 hours	44%	49%

decomposition over the remaining four days. In summary, the presence of the ether group in the synthetic (n-3)- β -oxa compound **84** does not alter the chemical stability of the compound **84** relative to arachidonic acid **3** because each compound undergoes autoxidation at a similar rate.

In a similar manner to the previous experiments with the β -oxa compounds **83** and **84**, a 1: 1 mixture of the synthetic (n-6)- β -thia compound **85** and arachidonic acid **3** was allowed to decompose by thin film autoxidation for various periods in the absence of additive, with 5% α -tocopherol **15** and with the radical initiator AIBN. The amounts of each fatty acid remaining were determined by HPLC and the conditions and results are summarised in Table 3.3.

In Table 3.3, following extensive decomposition using 20% AIBN or a week of autoxidation, the amounts of the two compounds **3** and **85** remaining differ by over 25%. Hence, the rate of oxidation of the β -thia compound **85** is more rapid than the rate of arachidonic acid **3** oxidation. The β -thia group must be responsible for the relative instability of the compound **85**, so the formation of a novel intermediate such as a resonance stabilised α -centred radical may lead to decomposition of the synthetic compound **85** via the breakdown of the β -thia group.

Table 3.3: Percentages of arachidonic acid **3** and [5'Z,8'Z,11'Z,14']-eicosatetraenylthioacetic acid **85** recovered following autoxidation

Autoxidation method:	20:4(n-6) 3	23:4(n-6)- β -thia 85
no additive 70 hours	98%	99%
no additive 7 days	68%	28%
5% α -tocopherol 15 60 hrs	98%	86%
20% AIBN 60 hrs	87%	57%

5% α -tocopherol **15** did not markedly alter the rate of PUFA oxidation, whereas 20% AIBN accelerated PUFA decomposition relative to the sample with no additive after 70 hours. During a week of autoxidation with no additive, the build up of unstable oxidation products over the first three days of autoxidation accelerated the rate of decomposition over the remaining four days.

In summary, the presence of the β -thia group in the synthetic compound **85** lowers the chemical stability of the compound **85** relative to arachidonic acid **3** as the synthetic compound **85** undergoes decomposition during autoxidation at a higher rate than arachidonic acid **3**.

A comparable set of experiments to those already performed with the synthetic compounds **83-85** was undertaken with the synthetic compound **86**. A 1 : 1 mixture of the synthetic γ -thia compound **86** and arachidonic acid **3** was allowed to decompose by thin film autoxidation for various periods in the absence of additive, with 5% α -tocopherol **15** and with the radical initiator AIBN. The amounts of each fatty acid remaining were determined by HPLC and the conditions and results are summarised in Table 3.4.

Under all the conditions used, in Table 3.4 the amounts of the two compounds **3** and **86** remaining are almost equal and less than ten percent of each compound decomposed in

Table 3.4: Percentages of arachidonic acid **3** and [5'Z,8'Z,11'Z,14'Z]-eicosatetraenylthioacetic acid **86** recovered following autoxidation

Autoxidation method	20:4(n-6) 3	24:4(n-6)- γ -thia 86
no additive 70 hours	101%	98%
no additive 7 days	102%	96%
5% α -tocopherol 60 hours	102%	92%
20% AIBN 60 hours	100%	96%

each case. The absence of decomposition was apparent under conditions which led to the decomposition of more than 50% of both arachidonic acid **3** and the synthetic PUFAs **83-85**. In particular, extensive decomposition of arachidonic acid **3** and the γ -thia compound **86** failed to occur using the radical initiator AIBN over 60 hours and using no additive during a week of autoxidation. Hence, the γ -thia functional group present in the synthetic compound **86** inhibits the autoxidation process. The compound **86** must be inhibiting the chain transfer process, therefore acting as a very effective antioxidant.

In summary, the presence of the γ -thia group in the synthetic compound **86** increases the chemical stability of the mixture of the compound **86** and arachidonic acid **3** relative to arachidonic acid **3** with the other synthetic compounds **83-85**.

Earlier measurements using proton nmr spectroscopy and HPLC found that a 20% yield and a 30% yield, respectively, of the HPETE isomers **12-14** and **44-46** is observed following autoxidation with 5% α -tocopherol **15** for 72 hours. By measuring the amount of arachidonic acid **3** remaining in a 1 : 1 mixture with one of the synthetic PUFAs **83-86** following autoxidation for 60 hours, it was found that less than 10% of the arachidonic acid **3** had been oxidised in each case. So in the mixtures studied in this Chapter, no more than a 10% yield of the HPETE isomers **12-14** and **44-46** is possible. As already mentioned, the rate of oxidation is accelerated by the decomposition of unstable oxidation products. The initial rate of oxidation is dependent on low levels of

these unstable products and on the initial levels of other minor impurities which accelerate the oxidation process. Consequently, variations in the levels of compounds which initiate the oxidation process are responsible for significant variations in the rate of oxidation and subsequent yields of products.

The HPLC assay applied above to the measurement of PUFA consumption during thin film autoxidation is potentially suitable for monitoring the consumption of arachidonic acid **3** and the synthetic fatty acids **83-86** during enzyme catalysed oxidation of a mixture of PUFAs. As discussed earlier, synthetic analogues of naturally occurring fatty acids and the oxidation products of these fatty acids have been shown to influence the activity of LO enzymes so similar effects were anticipated for the compounds **83-86**. Using the above HPLC assay with mixtures of arachidonic acid **3** and one of the synthetic compounds **83-86**, the oxidation of a synthetic substrate by LO and changes in the enzyme activity with respect to the oxidation of arachidonic acid **3** can potentially be demonstrated. With this application in mind, simple systems containing only the known substrate arachidonic acid **3** were used to develop a procedure to monitor fatty acid consumption by either 15-LO or 5-LO.

Conditions were investigated for monitoring the consumption of arachidonic acid **3** over time by 15-LO. Arachidonic acid **3** (0.4 mg / ml, 1 mM) and lauric acid (0.3 mg / ml) were incubated in phosphate buffer (0.1 M, pH 9.0) with varying amounts of 15-LO (2-100 µg / ml) in order to determine the appropriate concentration of 15-LO which achieves partial consumption of the arachidonic acid **3** over 10-20 minutes. Aliquots were taken during the incubation and the reaction was terminated by the addition of acid to denature the enzyme and triphenylphosphine to reduce the unstable HPETE isomers **12-14** and **44-46** to the alcohols **48-53**. The fatty acids were extracted into dichloromethane, the dichloromethane was evaporated, the residue was dissolved in mobile phase and analysed by injection onto the HPLC system described earlier.

Using a 15-LO concentration of 18 $\mu\text{g} / \text{ml}$, 30% of the arachidonic acid **3** was consumed after 12 minutes. A gradual decrease in the peak area for arachidonic acid **3** relative to lauric acid over time was observed and confirmed that LO catalysed oxidation was occurring. The consumption of arachidonic acid **3** was accompanied by a decrease in the area of the peak corresponding to triphenylphosphine ($R_T = 10.3$ minutes) and the formation of two new peaks corresponding to (15*S*)-HETE (*S*)-**53** and triphenylphosphine oxide with retention times of 4.3 and 5.3 minutes, respectively. To simplify the procedure, in subsequent experiments no reducing agent was used and the samples were analysed by HPLC on the day of the experiment to avoid PUFA decomposition resulting from the instability of hydroperoxide products.

Potato 5-LO was prepared from potato tubers as outlined earlier. The concentration of active enzyme in the crude protein extract was not determined. An incubation was performed to monitor the consumption of arachidonic acid **3** (1 mg / ml, 3 mM) by potato 5-LO in phosphate buffer (40 mM, pH = 6.0) using HPLC. The extraction procedure was the same as that employed above for 15-LO except that triphenylphosphine was not used. During extraction into the organic solvent, it was observed that the aliquots had a tendency to form emulsions with dichloromethane if vigorous stirring was used. To avoid this problem, samples were extracted using slow stirring for extended periods (30-60 minutes).

Substrate consumption was confirmed because 30% of the arachidonic acid **3** was consumed following incubation with a 20% by volume solution of 5-LO extract for 16 minutes. Whilst the exact concentration of 5-LO is unknown and the 5-LO activity was variable, in subsequent experiments a 5-25% solution of 5-LO extract in buffer was found to be sufficient to achieve partial consumption of arachidonic acid **3** (3 μM) in 10-15 minutes.

The HPLC assay was next used to monitor the incubation of arachidonic acid **3** with LO at a lower fatty acid concentration (90 μM). Using a range of volumes of 5-LO and 15-LO

stock solutions, the volume of enzyme required to achieve oxidation of 25-50% of the arachidonic acid **3** after 8 minutes was determined. A protein concentration of between 2-8 $\mu\text{g} / \text{ml}$ for 15-LO was found to be appropriate. A concentration of 90 μM is within the range of physiological concentrations known for arachidonic acid **3** so the assay can potentially be used to monitor the effects of known and potential LO inhibitors at fatty acid concentrations equal to those found *in vivo*.

For both 5-LO and 15-LO, in the systems described above the enzyme activity was found to vary with the preparation of each batch of buffer, the age of each stock solution of enzyme and with small changes in buffer pH. Before an experiment was performed using mixtures containing the synthetic PUFAs **83-86**, a preliminary experiment was necessary to gauge the enzyme activity of the LO stock solution. The consumption of arachidonic acid **3** (90 μM) after 8 minutes was monitored over a range of concentrations of 15-LO (1-20 $\mu\text{g} / \text{ml}$) or the crude 5-LO extract (15%-30% by volume). The aim of the preliminary experiment was to identify the volume of enzyme which resulted in consumption of 25-50% of the arachidonic acid **3** after 8 minutes in the control sample which contained only arachidonic acid **3** and the standard lauric acid. Under identical conditions, the consumption of arachidonic acid **3** by LO in samples which also contained one of the synthetic fatty acids **83-86** could then be compared to the control sample.

Experiments were performed to observe the effect of the synthetic fatty acids **83**, **84** and **86** on the consumption of arachidonic acid **3** (90 μM) by either soybean (S)-15-LO or potato (S)-5-LO. Six samples were used in each experiment with the mass ratio of synthetic compound to arachidonic acid **3** (90 μM) at 0 : 1, 0.25 : 1, 0.5 : 1, 1 : 1, 2 : 1 and 4 : 1. For each of the six mass ratios, samples were analysed by HPLC at 0, 8 and 16 minutes after addition of enzyme and the amount of fatty acid remaining in the latter samples was expressed as a percentage of the initial value.

The consumption of arachidonic acid **3** (90 μ M) in the presence of [9'Z,12'Z,15'Z]-octadecatrienyloxyacetic acid **84** by 5-LO crude extract (17% by volume) was monitored by HPLC and the results are summarised in Figs 3.3 and 3.4.

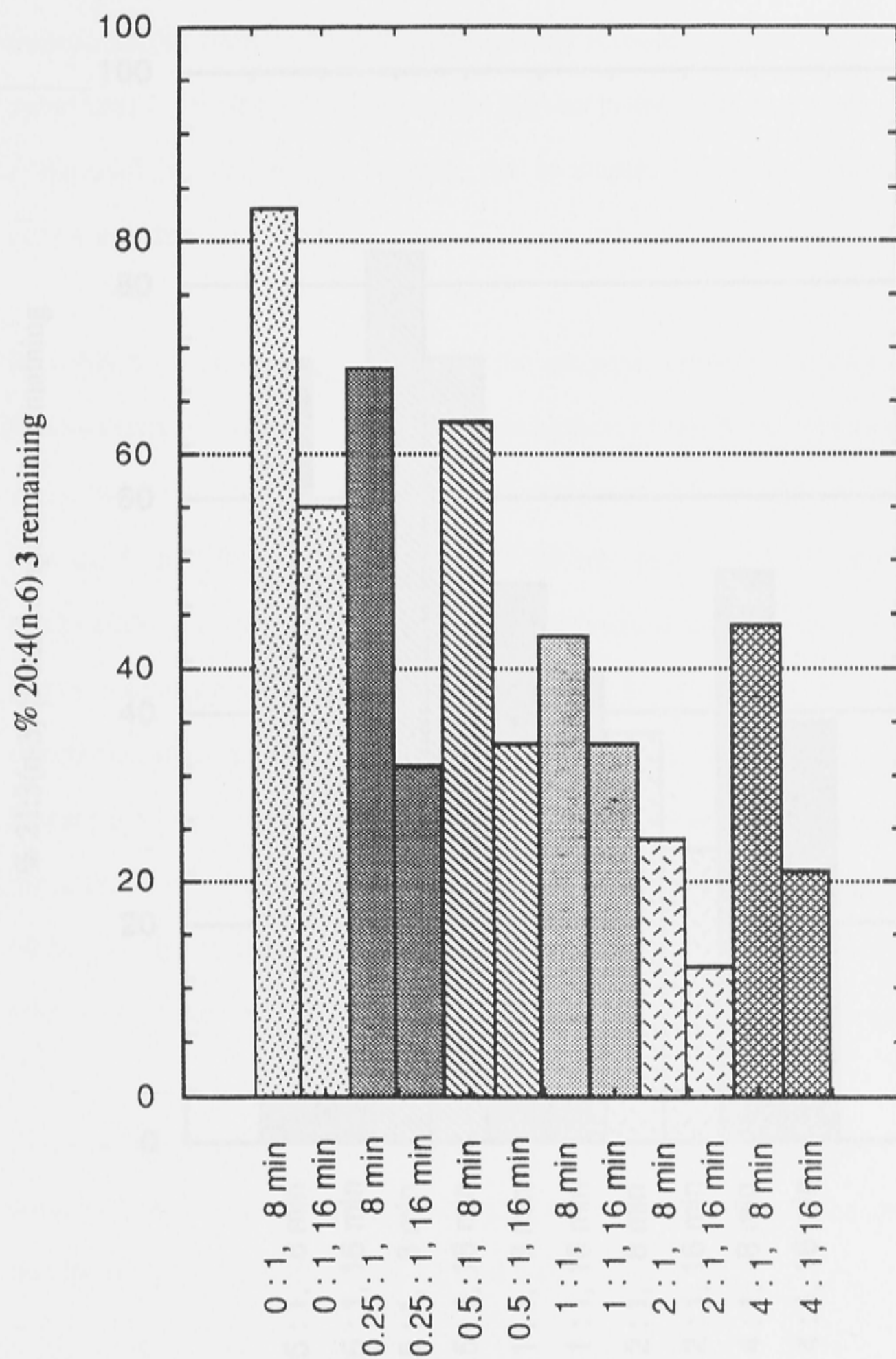
In Fig 3.3 the decrease in the amount of arachidonic acid **3** present over time indicates that compound **3** is consumed by 5-LO, as expected from the product study described in the previous Chapter. In Fig 3.4 the decrease in the amount of the (n-3)- β -oxa compound **84** present over time indicates that the compound **84** is also a substrate of 5-LO. When the mass ratio was 1 : 1, approximately equal amounts of the two compounds **3** and **84** remained after 8 or 16 minutes and hence the β -oxa compound **84** is consumed by 5-LO at a similar rate to arachidonic acid **3**.

The trend observed in Fig 3.3 for arachidonic acid **3** during incubation with 5-LO is that as the initial concentration of the (n-3)- β -oxa compound **84** was increased and the initial concentration of arachidonic acid **3** was unchanged, the percentage of arachidonic acid **3** remaining decreased after the same period of time. Hence, the rate of consumption of arachidonic acid **3** increases with increasing initial concentrations of the (n-3)- β -oxa compound **84**, so the presence of the synthetic compound **84** results in an increase in enzyme activity.

A trend is also observed for the consumption of the β -oxa compound **84**. In Fig 3.4, as the initial concentration of arachidonic acid **3** was held constant and the initial concentration of the β -oxa compound **84** was successively doubled, the percentage of the β -oxa compound **84** remaining decreased after the same period of time. Hence, the rate of consumption of the β -oxa compound **84** more than doubles with each doubling in concentration of the synthetic compound **84**.

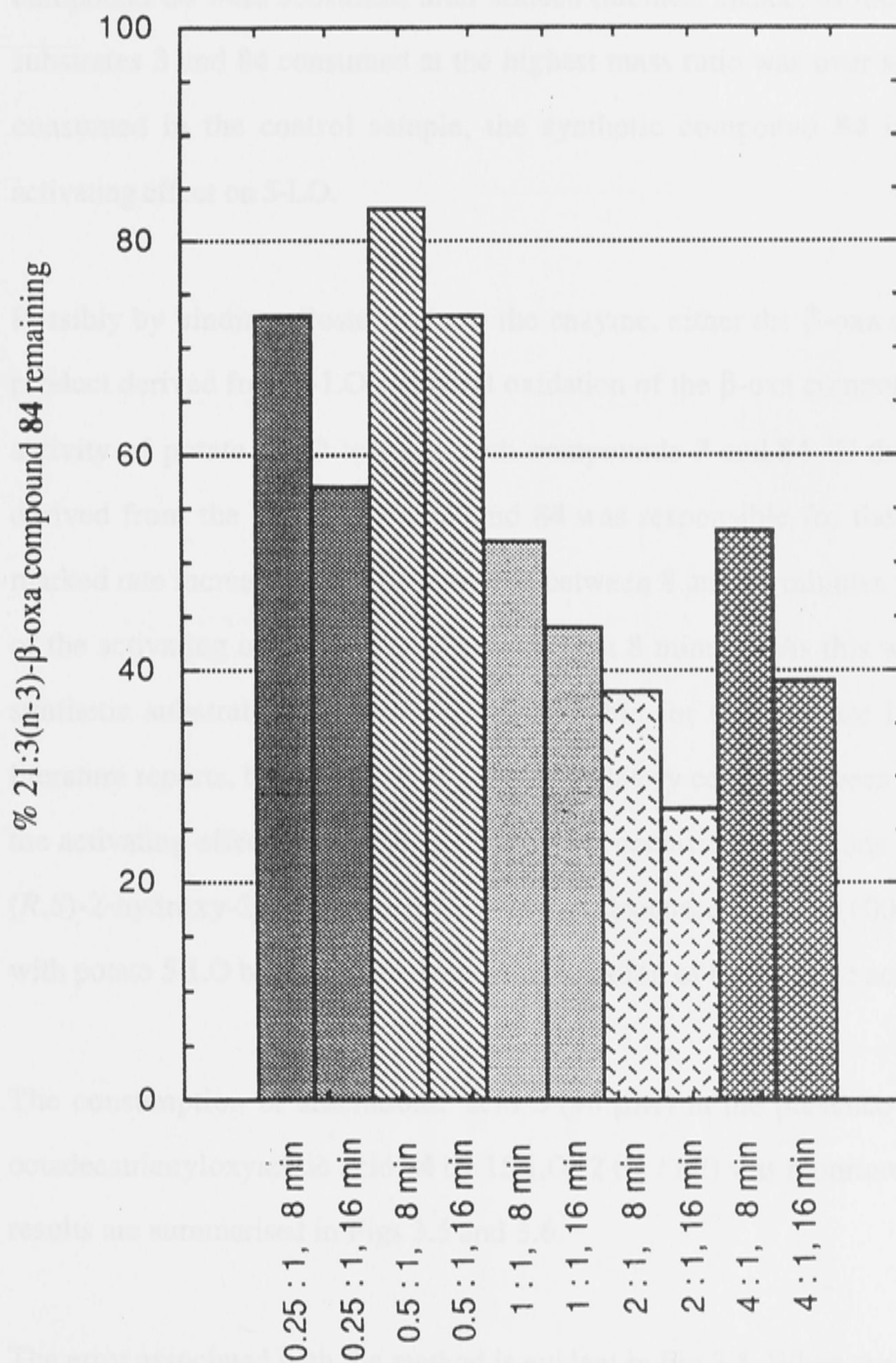
The Michaelis constant (K_m) is reported to be not more than 38 μ M for arachidonic acid **3** with potato 5-LO,^{60,97} which is less than half the concentration of arachidonic acid **3** used in the above experiment. In a system where the enzyme activity remains constant,

Fig 3.3: The consumption of arachidonic acid **3** (90 μ M) by 5-LO crude extract (17% v/v) in the presence of [9'Z, 12'Z, 15'Z]-octadecatrienyloxyacetic acid **84** (21-340 μ M)



Mass ratio : 21:3(n-3)- β -oxa **84** : 20:4(n-6) **3** / Time

Fig 3.4: The consumption of [9'Z, 12'Z, 15'Z]-octadecatrienyloxyacetic acid **84** (21-340 μ M) by 5-LO crude extract (17% v/v) in the presence of arachidonic acid **3** (90 μ M)



Mass ratio: 21:3(n-3)-β-oxa **84** : 20:4(n-6) **3** / Time

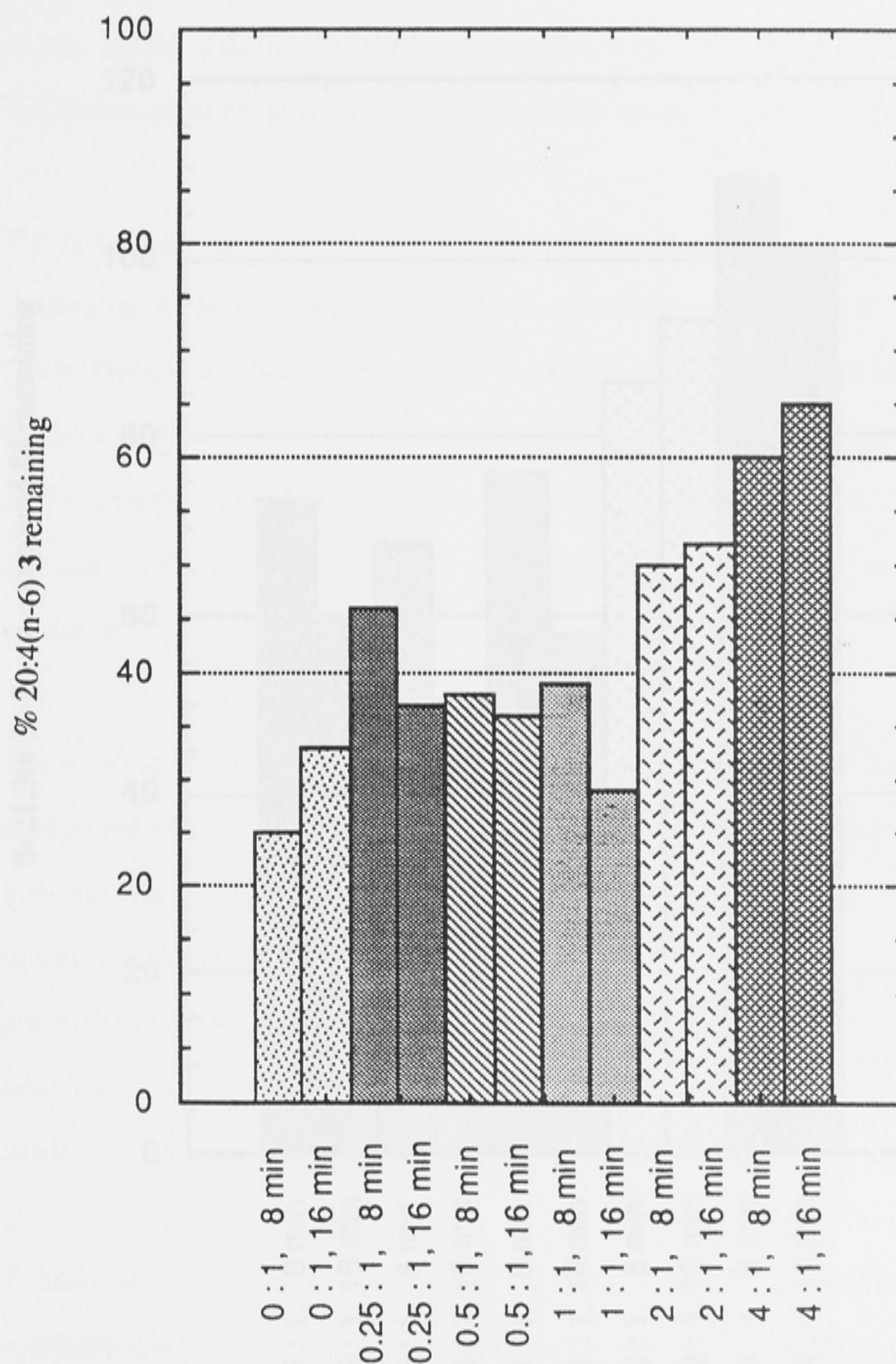
increasing the concentration of a substrate above double the K_m will not greatly alter the rate of reaction. In the control sample above, arachidonic acid **3** (90 μM) was the only substrate present and 40 μM of the compound **3** was consumed after sixteen minutes. At the highest mass ratio, 70 μM of the arachidonic acid **3** and 200 μM of the synthetic compound **84** were consumed after sixteen minutes. Hence, as the total amount of the substrates **3** and **84** consumed at the highest mass ratio was over six times the amount consumed in the control sample, the synthetic compound **84** is clearly having an activating effect on 5-LO.

Possibly by binding allosterically to the enzyme, either the β -oxa compound **84** or the product derived from 5-LO catalysed oxidation of the β -oxa compound **84** increases the activity of potato 5-LO towards both compounds **3** and **84**. If the oxidation product derived from the synthetic compound **84** was responsible for the activating effect, a marked rate increase would be observed between 8 and 16 minutes following formation of the activating oxidation product in the first 8 minutes. As this was not the case, the synthetic substrate **84** is probably responsible for the increase in 5-LO activity. In literature reports, binding to an allosteric regulatory centre has been proposed to explain the activating effect of analogues of naturally occurring fatty acids.^{101,104} For example, (*R,S*)-2-hydroxy-2-trifluoromethyl-(4*E*)-octadecenoic acid **81** (100 μM) does not react with potato 5-LO but increases the rate of oxidation of arachidonic acid **3** by the enzyme.

The consumption of arachidonic acid **3** (90 μM) in the presence of [9'*Z*,12'*Z*,15'*Z*]-octadecatrienyloxyacetic acid **84** by 15-LO (2 μg / ml) was monitored by HPLC and the results are summarised in Figs 3.5 and 3.6.

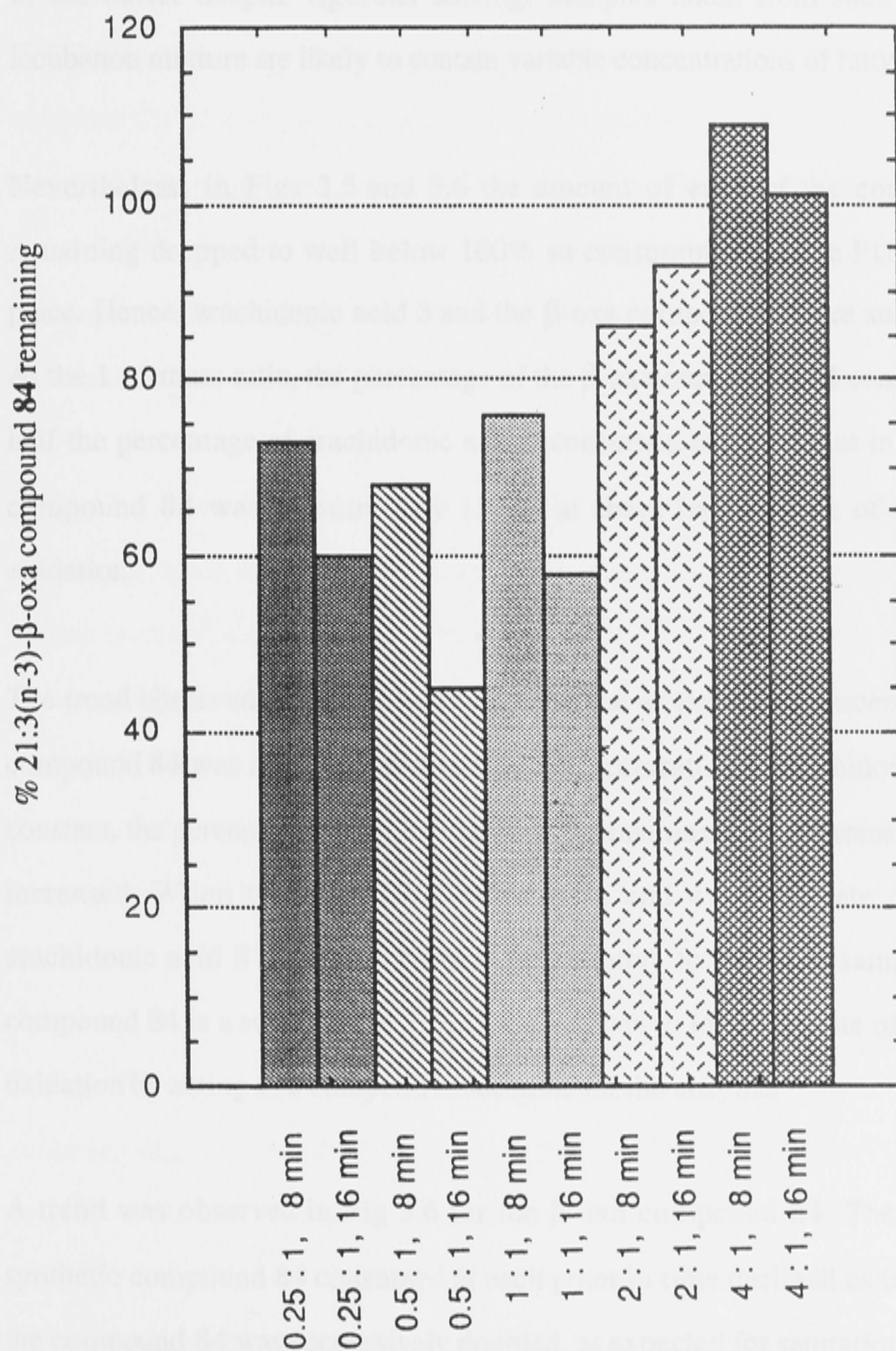
The error associated with the method is evident in Fig 3.5. When the mass ratio was 0 : 1 and 4 : 1, the amount of arachidonic acid **3** remaining after 16 minutes exceeded the amount present after 8 minutes, by 8% and 5%, respectively. Formation of the substrate **3** over time is very unlikely, so this outcome indicates experimental error and may partly result from the high variability in the integration of HPLC peaks. The variability is due to

Fig 3.5: The consumption of arachidonic acid **3** (90 μ M) by 15-LO (2 μ g / ml) in the presence of [9'Z, 12'Z, 15'Z]-octadecatrienyloxyacetic acid **84** (21-340 μ M)



Mass ratio : 21:3(n-3)-β-oxa **84** : 20:4(n-6) **3** / Time

Fig 3.6: The consumption of [9'Z, 12'Z, 15'Z]-octadecatrienyloxyacetic acid **84** (21-340 μ M) by 15-LO (2 μ g / ml) in the presence of arachidonic acid **3** (90 μ M)



Mass ratio: 21:3(n-3)-β-oxa **84** : 20:4(n-6) **3** / Time

the relatively broad peak shape observed for all fatty acids. Error was more frequently observed at the highest total concentrations of fatty acids and may also be a consequence of the poor solubility of each fatty acid in aqueous buffers. Poor fatty acid solubility was sometimes evident in reaction mixtures when oily globules were observed on the surface of the buffer despite vigorous stirring. Samples taken from such an inhomogenous incubation mixture are likely to contain variable concentrations of fatty acids.

Nevertheless, in Figs 3.5 and 3.6 the amount of each of the compounds **3** and **84** remaining dropped to well below 100% so consumption of the PUFAs **3** and **84** took place. Hence, arachidonic acid **3** and the β -oxa compound **84** are substrates for 15-LO. At the 1 : 1 mass ratio, the percentage of the β -oxa compound **84** consumed was close to half the percentage of arachidonic acid **3** consumed at each point in time, so the β -oxa compound **84** was consumed by 15-LO at about half the rate of arachidonic acid **3** oxidation.

The trend observed for arachidonic acid **3** is that as the initial concentration of synthetic compound **84** was increased and the initial concentration of arachidonic acid **3** remained constant, the percentage of arachidonic acid **3** remaining after the same period of time also increased. When the mass ratios were 2 : 1 and 4 : 1 the rate of consumption of arachidonic acid **3** successively decreased relative to the other samples. The synthetic compound **84** is a substrate for 15-LO and appears to lower the rate of arachidonic acid **3** oxidation by acting as a competitive substrate for the enzyme.

A trend was observed in Fig 3.6 for the β -oxa compound **84**. The percentage of the synthetic compound **84** consumed at each point in time declined as the concentration of the compound **84** was successively doubled, as expected for saturation of the enzyme.

Thus, in coincubation experiments with arachidonic acid **3** the β -oxa compound **84** promotes the oxidation of both compounds **3** and **84** by crude potato 5-LO and the compound **84** acts as a competitive substrate for both potato 5-LO and soybean 15-LO.

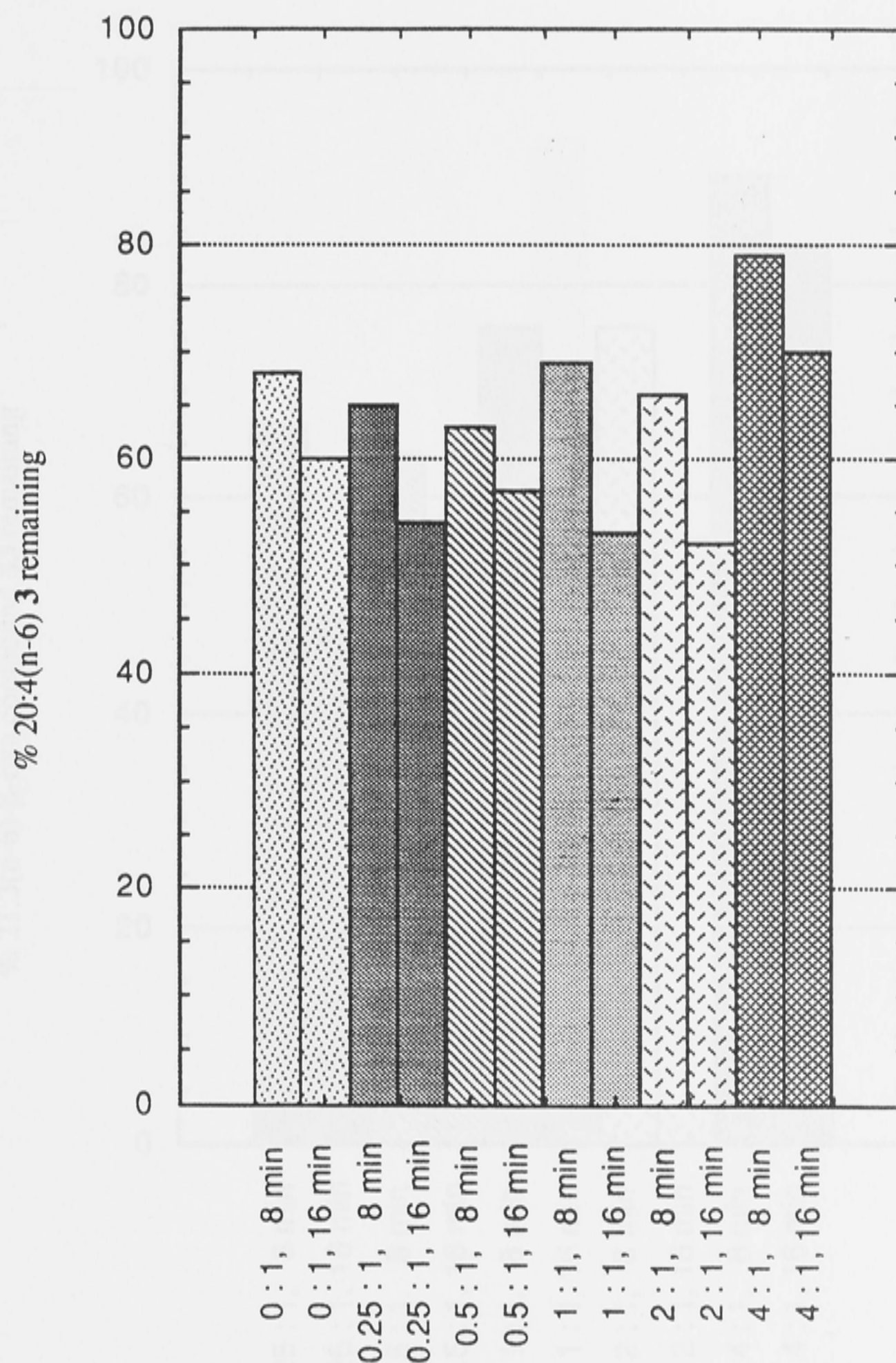
The results are presented for subsequent coincubation experiments of arachidonic acid **3** with the synthetic (n-6)- β -oxa compound **83** using soybean 15-LO in Figs 3.7 and 3.8, with the (n-6)- γ -thia compound **86** using potato 5-LO in Figs 3.9 and 3.10 and with the (n-6)- γ -thia compound **86** using soybean 15-LO in Figs 3.11 and 3.12.

From Figs 3.8, 3.10 and 3.12 it is evident that the (n-6)- β -oxa compound **83** is a substrate for 15-LO and that the (n-6)- γ -thia compound **86** is a substrate for both 5-LO and 15-LO because the percentages of the compounds **83** and **86** remaining have fallen below 100%. In each experiment, the oxidation of the synthetic compound **83** or **86** is accompanied by the oxidation of arachidonic acid **3** as the level of the naturally occurring substrate **3** falls below 100%, as shown in Figs 3.6, 3.8 and 3.10.

In Figs 3.7 and 3.8, at a 1 : 1 mass ratio the rates of consumption of arachidonic acid **3** and the synthetic compound **83** were approximately equal. In contrast with the properties characteristic of a competitive substrate, observed in Figs 3.5 and 3.6 for the (n-3)- β -oxa compound **84**, an activating effect on 15-LO is evident for the (n-6)- β -oxa compound **83** in Figs 3.7 and 3.8. Although the amount of arachidonic acid **3** consumed remained constant, the amount of the synthetic compound **83** consumed increased and consequently the total amount of the two PUFAs **3** and **83** oxidised by 15-LO increased as the mass ratio was increased.

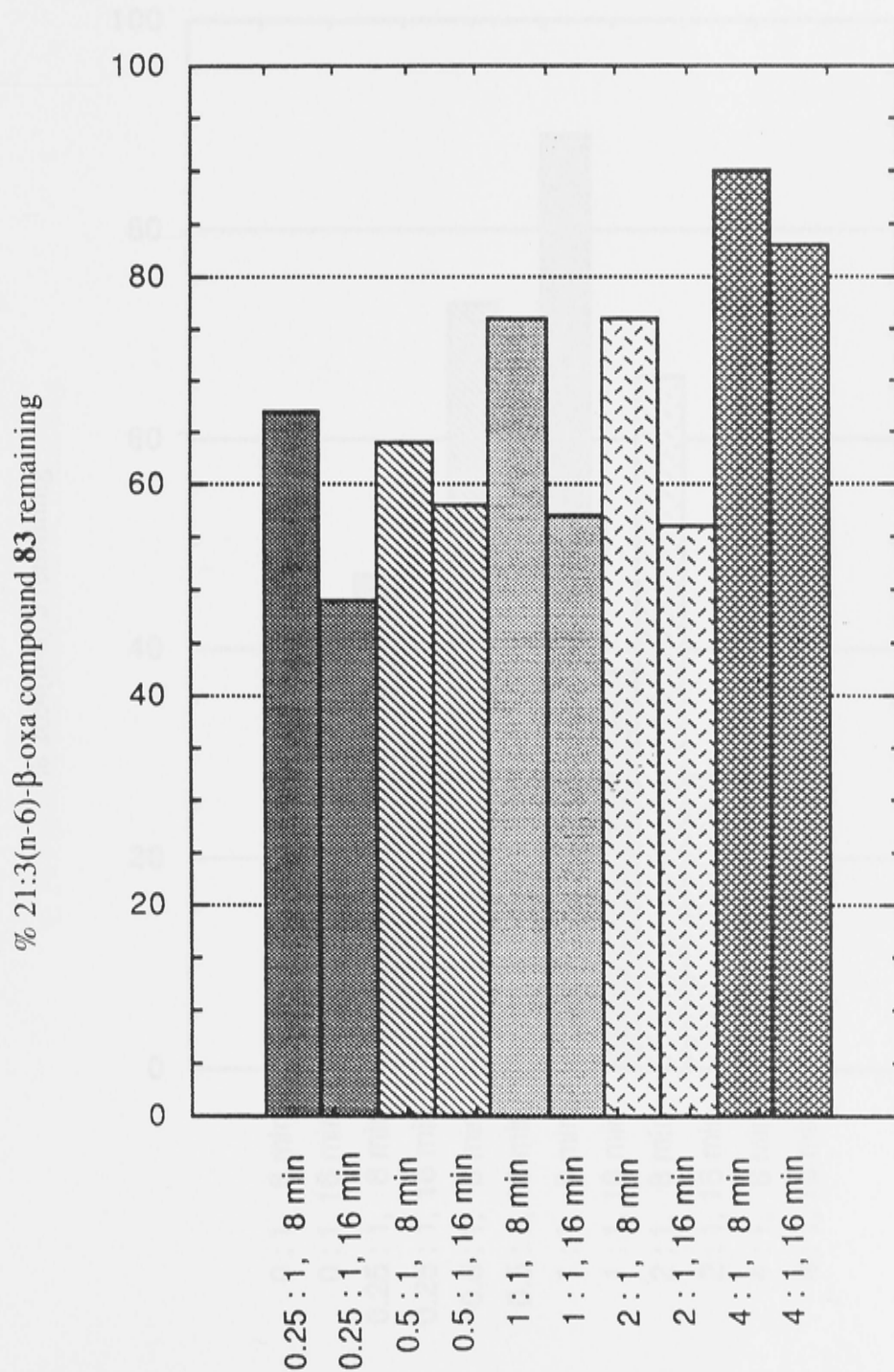
In Figs 3.9 and 3.10, at a 1 : 1 mass ratio the synthetic compound **86** was consumed more rapidly than arachidonic acid **3** by 5-LO. A notable increase was observed for the activity of the 5-LO extract towards the synthetic substrate **86** between 8 and 16 minutes as shown in Fig 3.9 in contrast with the corresponding 5-LO activity towards the (n-3)- β -oxa compound **84** shown in Fig 3.4. This behaviour may be a result of a variation in enzyme activity between different preparations of 5-LO extract. However, 5-LO inactivation caused by 5-HPETE **13** has been reported and inactivation of 5-LO was decreased by the antioxidant dithiothreitol,^{75,76} so the results in Figs 3.9 and 3.10 may be an example of the lowering of 5-LO inactivation. The antioxidant activity of the γ -thia

Fig 3.7: The consumption of arachidonic acid **3** (90 μ M) by 15-LO (2 μ g / ml) in the presence of [6'Z, 9'Z, 12'Z]-octadecatrienyloxyacetic acid **83** (21-340 μ M)



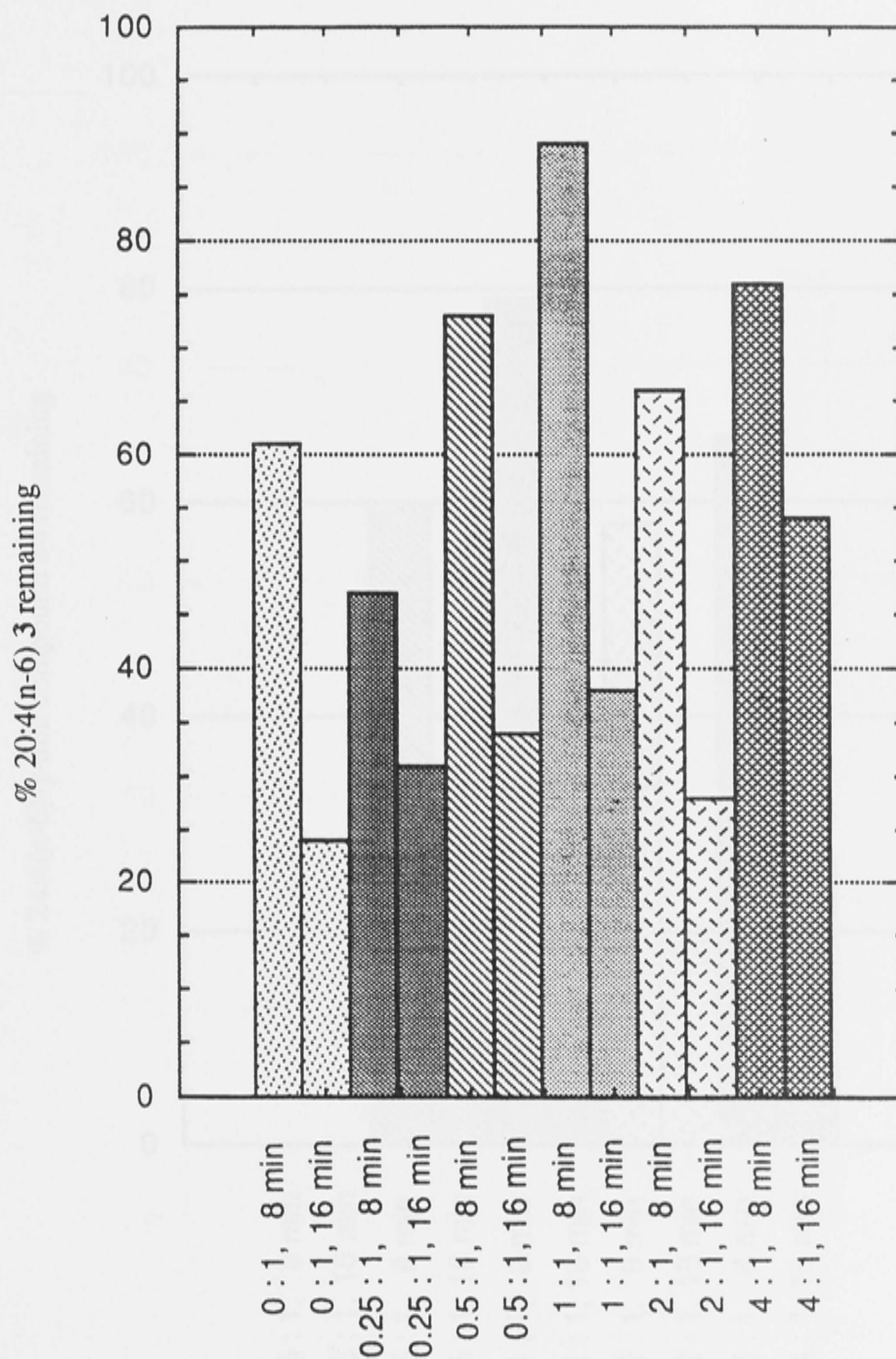
Mass ratio: 21:3(n-6)- β -oxa **83** : 20:4(n-6) **3** / Time

Fig 3.8: The consumption of [6'Z, 9'Z, 12'Z]-octadecatrienyloxyacetic acid **83** (21-340 μM) by 15-LO (2 μg / ml) in the presence of arachidonic acid **3** (90 μM)



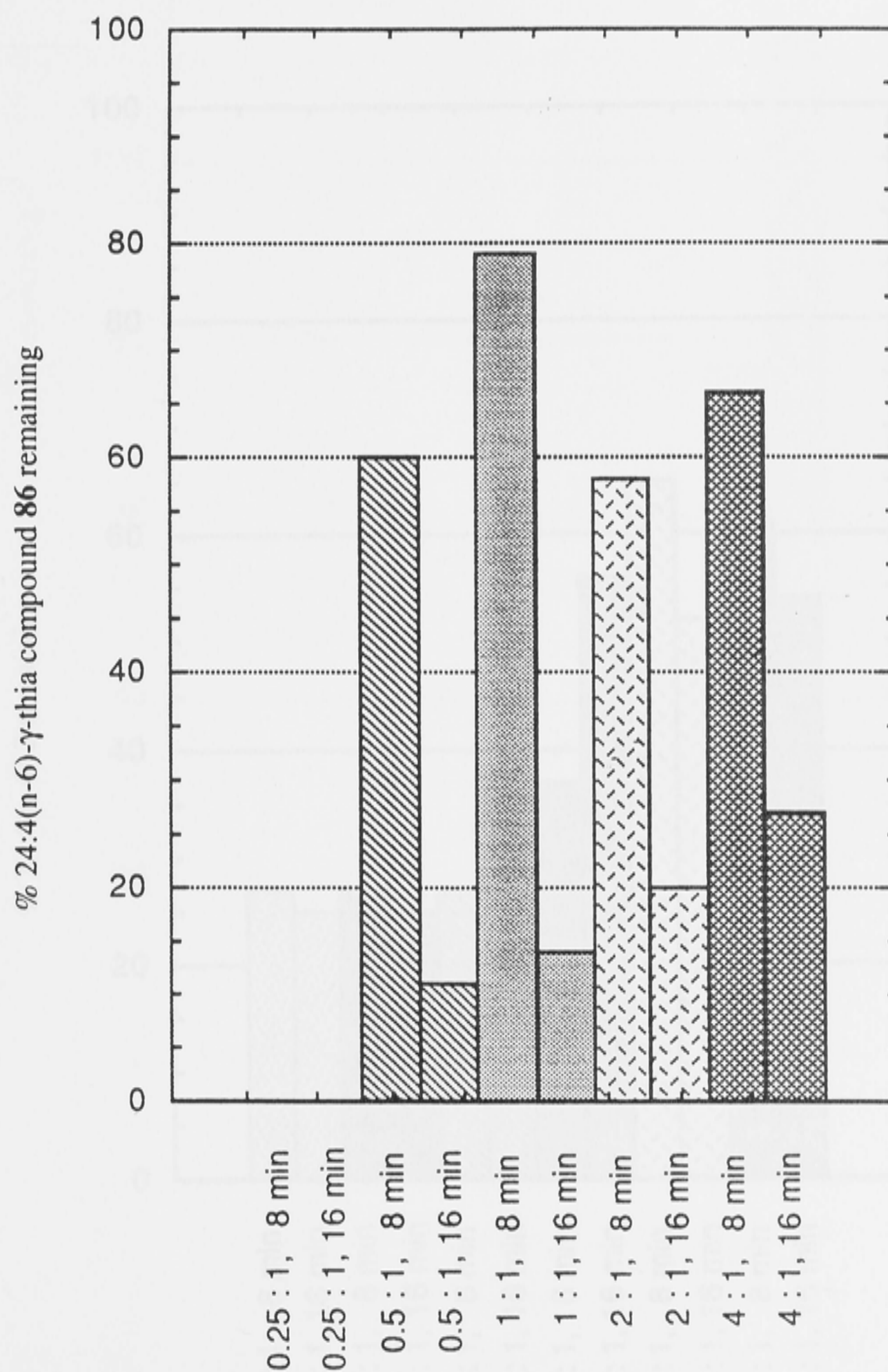
Mass ratio: 21:3(n-6)-β-oxa **83** : 20:4(n-6) **3** / Time

Fig 3.9: The consumption of arachidonic acid **3** (90 μM) by 5-LO (6% v/v) in the presence of [5'Z, 8'Z, 11'Z, 14'Z]-eicosatetraenylthiopropionic acid **86** (18-288 μM)



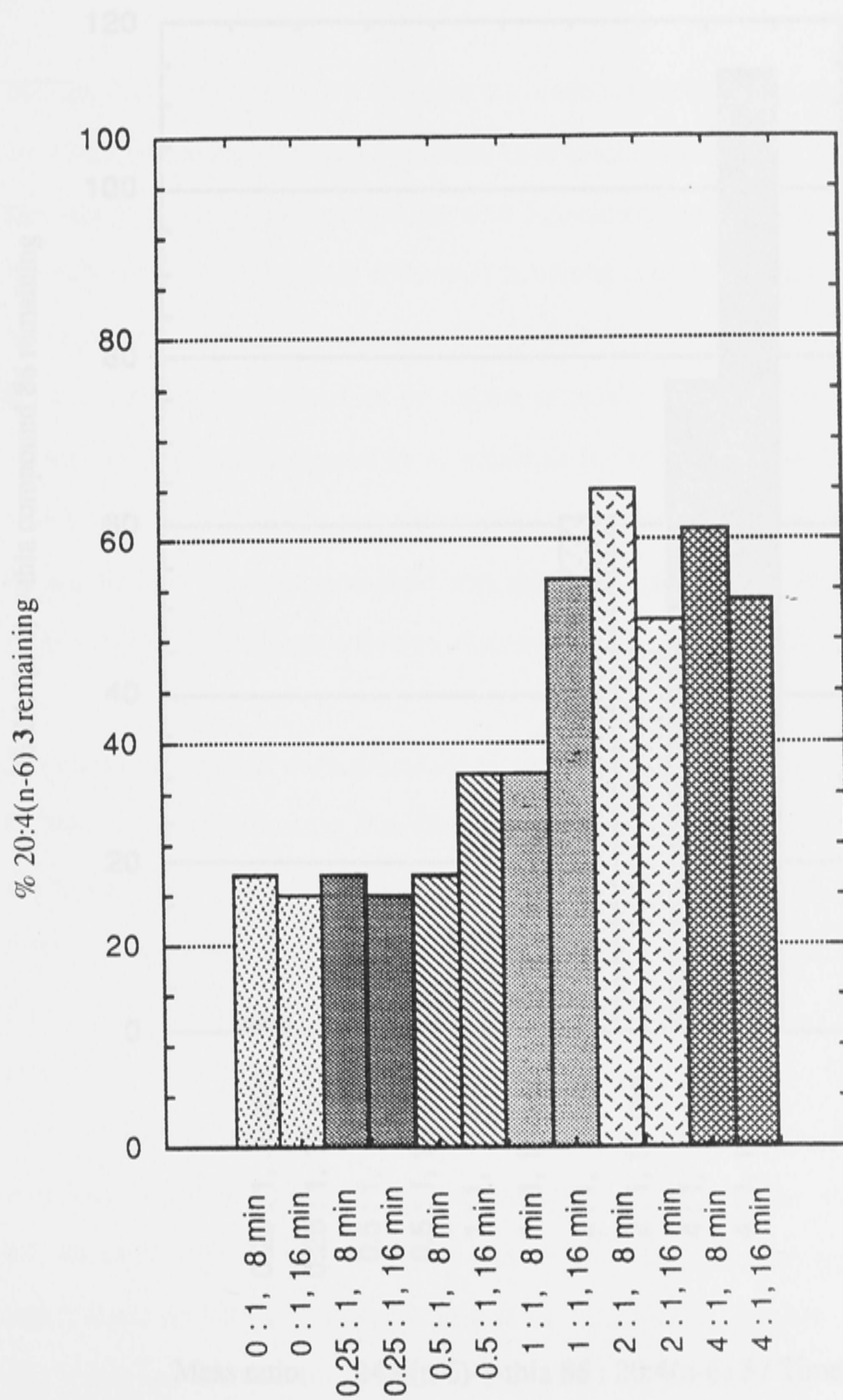
Mass ratio: 24:4(n-6)- γ -thia **86** : 20:4(n-6) **3** / Time

Fig 3.10: The consumption of [5'Z, 8'Z, 11'Z, 14'Z]-eicosatetraenylthiopropionic acid **86** (18-288 μ M) by 5-LO (6% v/v) in the presence of arachidonic acid **3** (90 μ M)



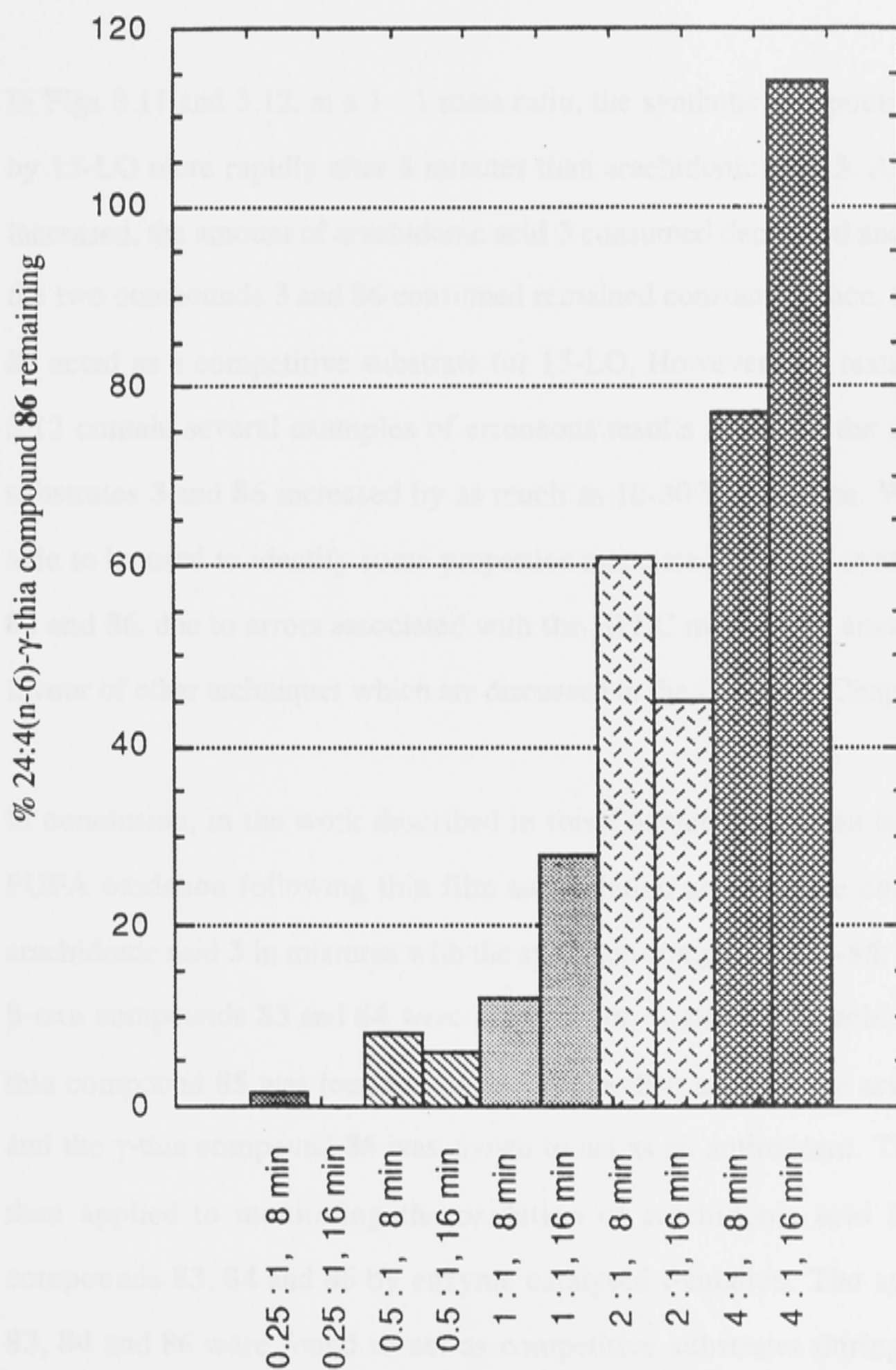
Mass ratio: 24:4(n-6)-γ-thia **86** : 20:4(n-6) **3** / Time

Fig 3.11: The consumption of arachidonic acid **3** (90 μ M) by 15-LO (8 μ g / ml) in the presence of [5'Z, 8'Z, 11'Z, 14'Z]-eicosatetraenylthiopropionic acid **86** (18-288 μ M)



Mass ratio: 24:4(n-6)- γ -thia **86** : 20:4(n-6) **3** / Time

Fig 3.12: The consumption of [5'Z, 8'Z, 11'Z, 14'Z]-eicosatetraenylthiopropionic acid **86** (18-288 μ M) by 15-LO (8 μ g / ml) in the presence of arachidonic acid **3** (90 μ M)



Mass ratio: 24:4(n-6)-γ-thia **86** : 20:4(n-6) **3** / Time

compound **86** was identified earlier in this Chapter and it may be this property that lowers the rate of 5-LO inactivation. In Figs 3.9 and 3.10, after 16 minutes the total amount of the substrates **3** and **86** consumed increased as the mass ratio was increased so the γ -thia compound **86** is responsible for an increase in the 5-LO activity.

In Figs 3.11 and 3.12, at a 1 : 1 mass ratio, the synthetic compound **86** was consumed by 15-LO more rapidly after 8 minutes than arachidonic acid **3**. As the mass ratio was increased, the amount of arachidonic acid **3** consumed decreased and the total amount of the two compounds **3** and **86** consumed remained constant. Hence, the γ -thia compound **86** acted as a competitive substrate for 15-LO. However, the results in Figs 3.11 and 3.12 contain several examples of erroneous results in which the concentration of the substrates **3** and **86** increased by as much as 10-30% with time. Whilst the assay was able to be used to identify some properties associated with the synthetic fatty acids **83**, **84** and **86**, due to errors associated with the HPLC method the assay was abandoned in favour of other techniques which are discussed in the following Chapters.

In conclusion, in the work described in this Chapter HPLC has been used to monitor PUFA oxidation following thin film autoxidation and enzyme catalysed oxidation of arachidonic acid **3** in mixtures with the synthetic compounds **83-86**. By autoxidation, the β -oxa compounds **83** and **84** were shown to be as stable as arachidonic acid **3**. The β -thia compound **85** was found to be less stable than arachidonic acid **3** by autoxidation and the γ -thia compound **86** was shown to act as an antioxidant. The HPLC assay was then applied to monitoring the oxidation of arachidonic acid **3** and the synthetic compounds **83**, **84** and **86** by enzyme catalysed oxidation. The synthetic compounds **83**, **84** and **86** were found to act as competitive substrates during coincubation with arachidonic acid **3** using soybean 15-LO. In addition, the synthetic compounds **84** and **86** were found to act as competitive substrates during coincubation with arachidonic acid **3** using potato 5-LO extract. Activating effects were observed during coincubation experiments with arachidonic acid **3** using 5-LO with the (n-3)- β -oxa compound **84** or

the (n-6)- γ -thia compound **86**. Activating effects were also observed using 15-LO in the coincubation of arachidonic acid **3** with the (n-6)- β -oxa compound **83**.

The aim of the research presented in this section was to investigate whether products formed from the synthetic PUFA **83-89** by LO catalyzed oxidation are comparable to results with those for the naturally occurring polyunsaturated fatty acids **1** and **5**. As less than 100 μ g of each substrate is required for spectroscopic analysis, this technique makes it possible to screen potential substrates using small samples of potential substrates.

In the literature, it has been commonly assumed that the conjugated dienes are equal for the conjugated *E,Z*-dienes formed by oxidation of the ω -6 and ω -3 arachidonic acid or docosahexaenoic acid **5**.^{14,15,16} By labeling the substrates with a specific isotope, the coefficient of a particular product, the rate of product formation can be determined based on the rate of change in absorbance and the appropriate yield can be calculated based on the

Results and Discussion: Chapter 3

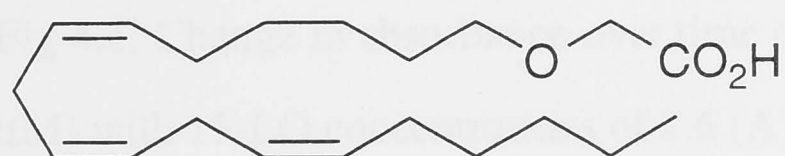
An ultraviolet spectroscopic study of β -oxa and β - and γ -thia fatty acid oxidation

The products formed from arachidonic acid **3** by LO enzymes, the HPETE regioisomers **12-14** and **44-46**, contain a conjugated *E,Z*-diene group. This functional group is a chromophore ($\lambda_{\text{max}} = 232\text{-}237\text{ nm}$) and can be used to spectroscopically monitor product formation by LO enzymes. Under identical conditions with different substrates the change in absorbance over time can be used to monitor aspects of the enzyme catalysed reaction such as the relative rate of product formation and the yields of products.

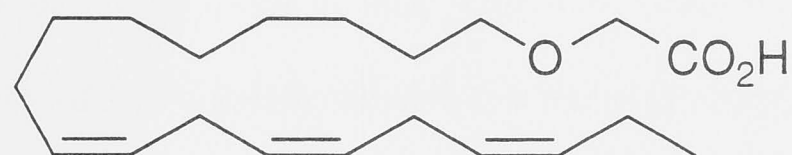
In Chapter 1 the HPETE regioisomers **12-14** and **44-46** formed from arachidonic acid **3** were identified by derivatisation to the HETE isomers **48-53**, purification by HPLC and characterisation by mass spectrometry. In Chapter 2, the oxidation of arachidonic acid **3** over time by either autoxidation or enzyme catalysed oxidation was confirmed using reversed phase HPLC. In the presence of arachidonic acid **3**, the synthetic compounds **84** and **86** were identified as competitive substrates for potato (*S*)-5-LO and the synthetic compounds **83**, **84** and **86** were identified as competitive substrates for soybean (*S*)-15-LO.

The aim of the research presented in this section was to determine whether products formed from the synthetic PUFAs **83-89** by LO catalysed oxidation and to compare the results with those for the naturally occurring substrates arachidonic acid **3** and docosahexaenoic acid **5**. As less than 100 μg of each substrate is required for spectroscopic analysis, this technique makes it possible to assess product formation using small samples of potential substrates.

In the literature, it has been commonly assumed that the extinction coefficients are equal for the conjugated *E,Z*-dienes formed by oxidation of linolenic acid **6**, arachidonic acid **3** or docosahexaenoic acid **5**.^{31,53,116} By taking into account the likely extinction coefficient of a particular product, the rate of product formation can be calculated based on the rate of change in absorbance and the approximate yield can be calculated based on the



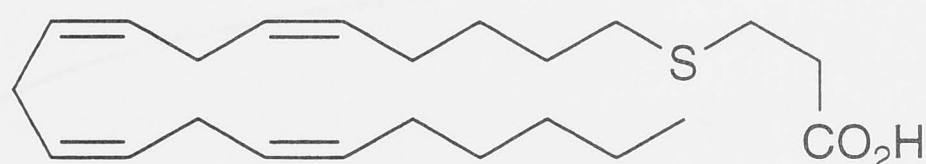
83



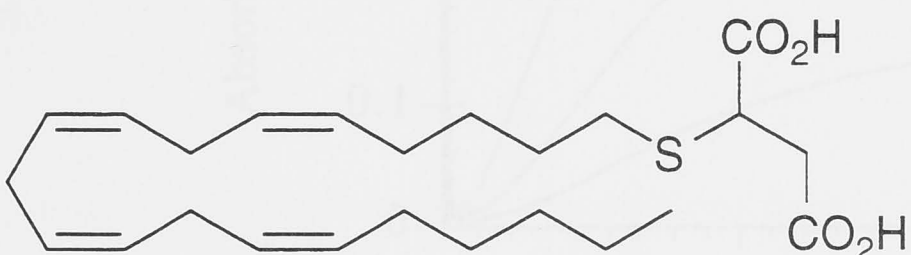
84



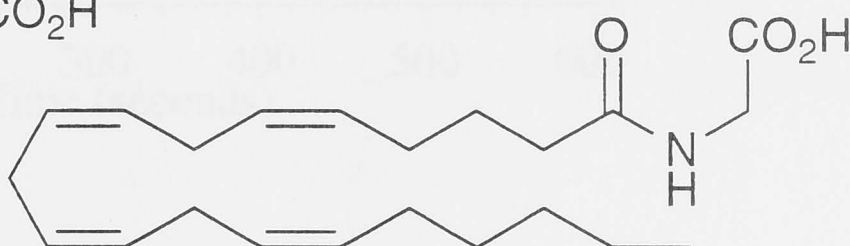
85



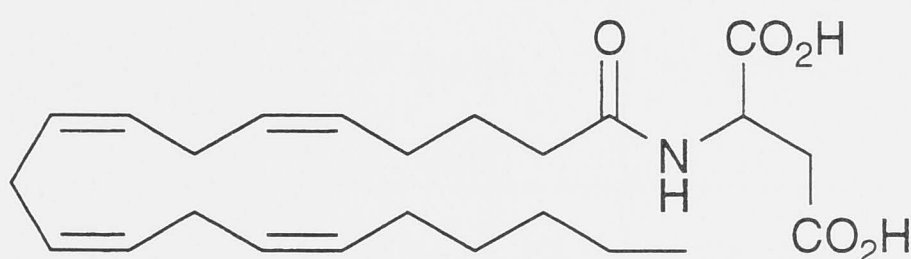
86



87



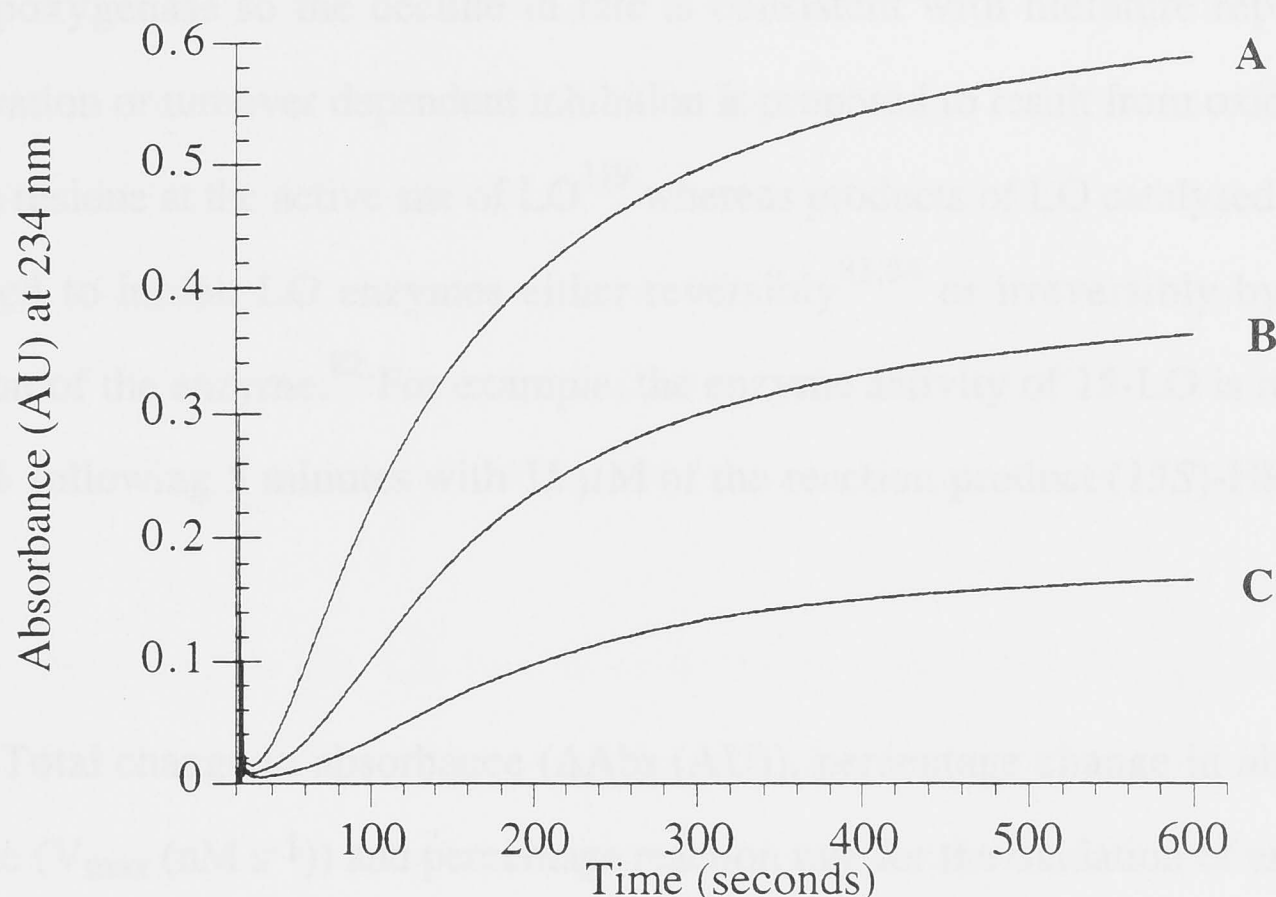
88



89

total change in absorbance. Precise determinations of molar extinction coefficients for the conjugated *E,Z*-diene containing oxidation products formed from triene fatty acids ($\epsilon = 22500\text{--}28000 \text{ M}^{-1} \text{ cm}^{-1}$)^{21,28} and tetraene fatty acids ($\epsilon = 23000\text{--}31000 \text{ M}^{-1} \text{ cm}^{-1}$)^{29,32,37,117} indicate a lack of agreement on whether a real difference exists between the extinction coefficients of different products. In this section, the maximum rates of product formation were calculated assuming that the molar extinction coefficient of each product is equal to the value recorded for (15*S*)-HPETE (**S**)-**14**. Near quantitative conversion of the arachidonic acid **3** (50 μM) to (15*S*)-HPETE (**S**)-**14** was achieved by incubation with 15-LO (50 $\mu\text{g} / \text{ml}$).¹¹⁷ From two readings of the change in

Fig 4.1: Change in absorbance over time observed for reaction of arachidonic acid **3** (20 μM) with 15-LO concentrations of 1.6 (**A**), 0.8 (**B**) and 0.4 $\mu\text{g} / \text{ml}$ (**C**)



absorbance with quantitative formation of (15*S*)-HPETE (**S**)-**53**, the observed extinction coefficient was $33000 \text{ M}^{-1} \text{ cm}^{-1}$ which is slightly higher than the reported values.

The dependence of the enzyme catalysed reaction on the concentration of enzyme was investigated by monitoring the change in absorbance over time for arachidonic acid **3** (20 μM) with three concentrations of 15-LO (1.6, 0.8 and 0.4 $\mu\text{g} / \text{ml}$) as shown in Fig 4.1. The total change in absorbance due to conjugated diene formation and the maximum rate of change in absorbance at different concentrations of 15-LO are shown in Table 4.1 for two experiments performed two hours apart. The change in absorbance was 1.67 AU when quantitative oxidation of 50 μM arachidonic acid **3** took place. Based on this result, within 10 minutes at the highest concentration of 15-LO in Fig 4.1 85% of the arachidonic acid **3** present is converted to (15*S*)-HPETE (**S**)-**53**.

At the lower two concentrations of 15-LO (0.4 and 0.8 $\mu\text{g} / \text{ml}$) it is evident that the reaction rate has declined after 10 minutes although there is still unutilised substrate **3** remaining in solution. Both product inhibition and self-inactivation have been reported for soybean lipoxygenase so the decline in rate is consistent with literature reports.^{82,118} Self-inactivation or turnover dependent inhibition is proposed to result from oxidation of a methionine residue at the active site of LO¹¹⁹ whereas products of LO catalysed oxidation are proposed to inhibit LO enzymes either reversibly^{83,84} or irreversibly by covalent modification of the enzyme.⁸² For example, the enzyme activity of 15-LO is reported to fall by 50% following 5 minutes with 15 μM of the reaction product (15*S*)-HPETE (*S*)-**53**.^{82,83}

Table 4.1: Total change in absorbance (ΔAbs (AU)), percentage change in absorbance, reaction rate (V_{max} (nM s^{-1})) and percentage reaction rate for the oxidation of arachidonic acid **3** (20 μM) by several concentrations of 15-LO ([15-LO] ($\mu\text{g} / \text{ml}$))

[15-LO] ($\mu\text{g} / \text{ml}$)	ΔAbs (AU)	% ΔAbs	V_{max} (nM s^{-1})	% V_{max}
0.4	0.16, 0.17	28, 29%	29, 28	17, 17%
0.8	0.35, 0.35	60, 60%	75, 77	49, 51%
1.6	0.56, 0.58	97, 100%*	149, 152	97, 100%*

* arbitrarily assigned as 100%

For 15-LO, the background absorbance due to the 15-LO stock solution (66 $\mu\text{g} / \text{ml}$) was small so that at the volumes of stock solution used (15, 30 and 60 μl in 2.5 ml buffer) the initial absorbance was unchanged. As shown in Table 4.1 for arachidonic acid **3** (20 μM) with 15-LO (0.4-1.6 $\mu\text{g} / \text{ml}$), the total change in absorbance and the maximum rate of change in absorbance both approximately doubled as the concentration of 15-LO was doubled. Although the results were readily reproduced under identical conditions with the same batches of buffer and enzyme solution, overnight refrigeration of enzyme stock

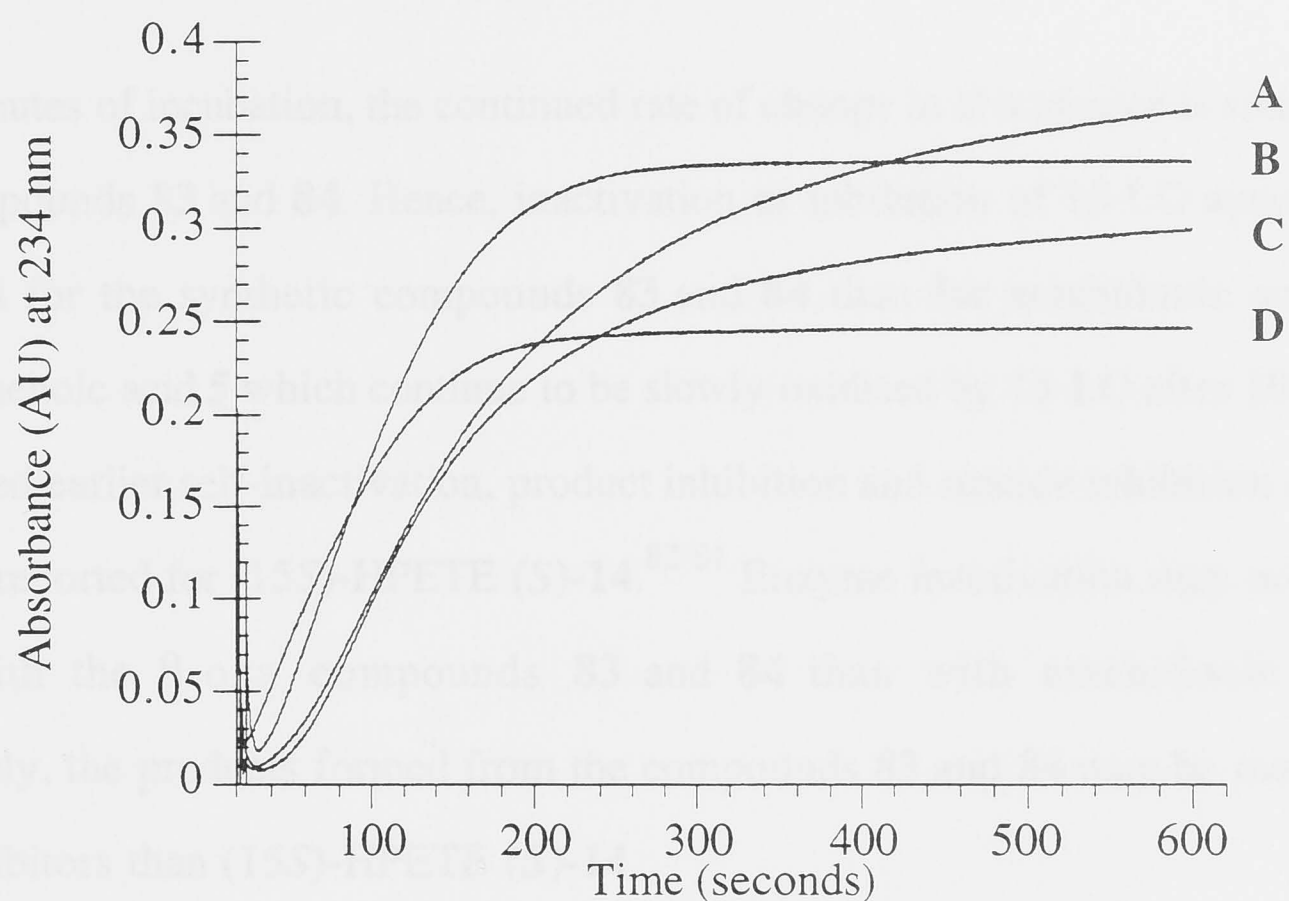
solution and different batches of stock solution resulted in significant changes in the observed activity of 15-LO.

The incubation of 15-LO with the synthetic compounds **83-89** was compared with arachidonic acid **3** and docosahexaenoic acid **5** by monitoring the change in absorbance over time. Arachidonic acid **3** (20 μ M) in buffer (pH = 9.0, 2.47 ml) was incubated with 15-LO (2 μ g in 30 μ l) for 10 minutes and the change in absorbance over time was monitored at 234 nm. The compounds **5** and **83-89** were monitored under identical conditions with the 15-LO concentration at 0.8 μ g / ml. The changes in absorbance over time are shown in Figs 4.2, 4.3 and 4.4 and the results are summarised in Table 4.2.

The results are shown in Fig 4.2, for the (n-6)- β -oxa compound **83**, the (n-3)- β -oxa compound **84** and the known substrates arachidonic acid **3** and docosahexaenoic acid **5**. In the previous Chapter it was shown that the (n-6)- β -oxa compound **83** and the (n-3)- β -oxa compound **84** were substrates for 15-LO when coincubated with arachidonic acid **3**. The above experiment indicates that when the β -oxa compounds **83** and **84** were individually incubated with 15-LO in the absence of a known substrate a product containing a conjugated *E,Z*-diene moiety is formed. This is consistent with the observation described in Chapter 2 that compounds **83** and **84** are substrates for 15-LO in the presence of arachidonic acid **3**, but it does not necessarily follow automatically. As discussed in the Introduction, a compound oxidised by 15-LO in the presence of a known substrate will not definitely be a substrate under similar conditions in the absence of the known substrate. For example, the furan fatty acid (10*Z*,12*Z*)-10,13-epoxyoctadecadienoic acid **70** is only oxidised by 15-LO when it is present with an equimolar amount of linoleic acid **4**.⁹⁴

Based on the maximum rate of change in absorbance, the maximum rate of reaction is higher for the β -oxa compounds **83** and **84** than for the endogenous substrates **3** and **5**. The PUFAs with the structures 18:3(n-6) **4** and 18:3(n-3) share the same 17-carbon lipophilic tail as the (n-6)- β -oxa compound **83** and (n-3)- β -oxa compound **84**,

Fig 4.2: Change in absorbance over time for reaction of the β -oxa compounds **83** (**B**) and **84** (**D**), arachidonic acid **3** (**A**) and docosahexaenoic acid **5** (**C**) with 15-LO (0.8 μg / ml)



respectively, and are oxidised by soybean 15-LO at 50% and 103% of the rate of arachidonic acid **3** oxidation.⁵³ The compound of similar chain length to the β -oxa compounds **83** and **84**, 21:4(n-6) is oxidised at 60% of the rate of arachidonic acid **3** oxidation.⁵³ Hence, the maximum rate of oxidation appears to be higher as a result of the presence of the oxygen in the compounds **83** and **84**.

The time taken to achieve the maximum rate of reaction or lag time is about 40 seconds longer for the naturally occurring substrates **3** and **5** than for the β -oxa compounds **83** and **84**. The lag time is proposed to be the time taken for an equimolar amount of fatty acid hydroperoxide to form and then oxidise the iron at the enzyme active site from the ferrous form to the ferric form, resulting in the active form of 15-LO.⁵⁵ The lag time can be abolished by the addition of an equimolar amount of fatty acid hydroperoxide to the

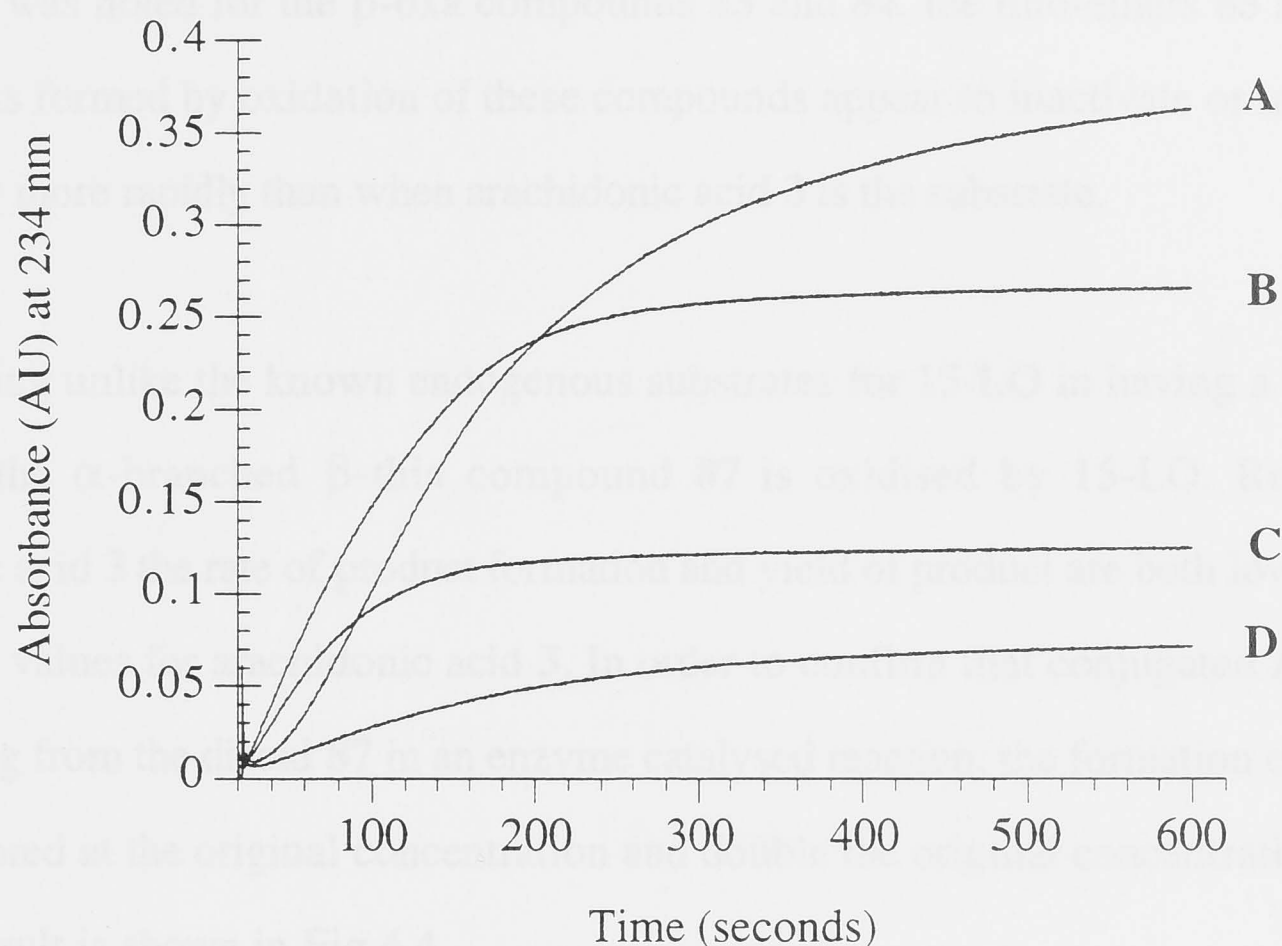
enzyme in buffer for several minutes prior to the addition of the fatty acid substrate. The synthetic compounds **83** and **84** decrease the lag time so they probably accelerate the oxidation of the protein bound iron possibly by forming hydroperoxide, binding to and oxidising the iron with a greater efficiency than the endogenous substrates **3** and **5**. However, the exact reason for the decrease in lag time is unclear.

After 4 minutes of incubation, the continued rate of change in absorbance is small for the β -oxa compounds **83** and **84**. Hence, inactivation or inhibition of 15-LO appears to be more rapid for the synthetic compounds **83** and **84** than for arachidonic acid **3** and docosahexaenoic acid **5** which continue to be slowly oxidised by 15-LO after 10 minutes. As discussed earlier self-inactivation, product inhibition and suicide inhibition of 15-LO have been reported for (15*S*)-HPETE (*S*)-**14**.⁸²⁻⁸⁴ Enzyme inactivation may occur more rapidly with the β -oxa compounds **83** and **84** than with arachidonic acid **3**. Alternatively, the products formed from the compounds **83** and **84** may be more potent 15-LO inhibitors than (15*S*)-HPETE (*S*)-**14**.

The above results indicate that compared with arachidonic acid **3**, soybean 15-LO oxidises (n-3) and (n-6) PUFAs with the β -oxa functionality more rapidly and with a shorter lag time but enzyme inhibition or inactivation occurs more rapidly than with the naturally occurring substrates **3** and **5**.

The oxidation of the synthetic substrates containing sulphur **85-87** by 15-LO was investigated next and the results are shown in Fig 4.3. In the previous Chapter coincubation experiments indicated that the γ -thia compound **86** was consumed more rapidly than arachidonic acid **3** using 15-LO. In Fig 4.3, it is immediately evident that the synthetic compounds **85-87** are all substrates for 15-LO because a change in absorbance occurred with time in each case. When incubated individually, the γ -thia compound **86** is nearly as good a substrate as arachidonic acid **3** because the maximum rates of product formation are close to equal for the two compounds **3** and **86** and based on the total change in absorbance about 70% of the yield of conjugated *E,Z*-diene from arachidonic

Fig 4.3: Change in absorbance over time for reaction of the β -thia compound **85** (C), the γ -thia compound **86** (B), the α -branched β -thia compound **87** (D) and arachidonic acid **3** (A) with 15-LO (0.8 $\mu\text{g} / \text{ml}$)



acid **3** is observed for the synthetic compound **86**. In contrast, as both the change in absorbance and maximum rate of reaction of the β -thia compound **85** are approximately half the values seen for the γ -thia compound **86**, the compound **85** is a markedly less suitable substrate for 15-LO than the γ -thia compound **86**. The higher yield of product formed from the γ -thia compound **86** relative to the β -thia compound **85** may result from a difference in the incidence of enzyme self-inactivation in the two cases. As self-inactivation of LO enzymes is reported to result from oxidation of a methionine residue at the enzyme active site,¹¹⁹ the antioxidant nature of the γ -thia compound **86** noted in the previous Chapter may lower the incidence of inactivation *via* this mechanism.

Like the result with the β -oxa compounds **83** and **84**, the lag times observed for reactions of the β -thia compound **85** and the γ -thia compound **86** are about 40 seconds

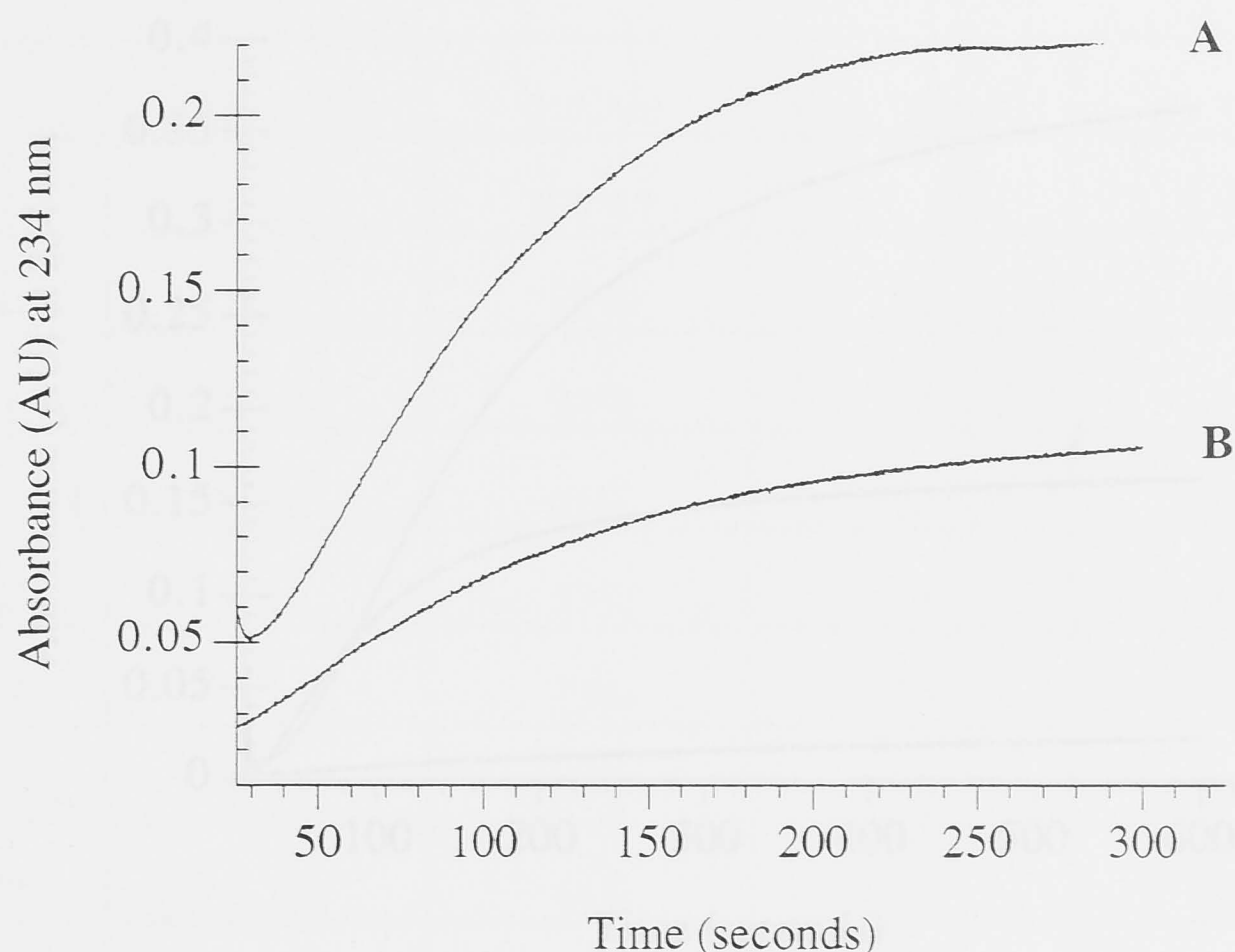
less than for arachidonic acid **3**. Again the synthetic compounds **85** and **86** may accelerate oxidation of the ferrous ion at the enzyme active site to produce the active form of the enzyme more rapidly than with arachidonic acid **3**. Whereas arachidonic acid **3** continued to undergo oxidation after 10 minutes with 15-LO, product stopped forming from the straight chain thio-ethers **85** and **86** after less than 5 minutes incubation with 15-LO. As was noted for the β -oxa compounds **83** and **84**, the thio-ethers **85** and **86** or the products formed by oxidation of these compounds appear to inactivate or inhibit 15-LO activity more rapidly than when arachidonic acid **3** is the substrate.

Despite being unlike the known endogenous substrates for 15-LO in having a branched structure, the α -branched β -thia compound **87** is oxidised by 15-LO. Relative to arachidonic acid **3** the rate of product formation and yield of product are both low at about 20% of the values for arachidonic acid **3**. In order to confirm that conjugated *E,Z*-diene was forming from the diacid **87** in an enzyme catalysed reaction, the formation of product was monitored at the original concentration and double the original concentration of 15-LO. The result is shown in Fig 4.4.

From Fig 4.4, the total change in absorbance and the maximum rate of product formation approximately doubled as the 15-LO concentration was doubled. The increase in change in absorbance and increase in the maximum rate for the diacid **87** with double the concentration of 15-LO is similar to the result observed using the substrate arachidonic acid **3** shown in Fig 4.1. Hence, the β -thia diacid **87** is also a substrate for soybean 15-LO but the rate of product formation and yield of product are relatively low.

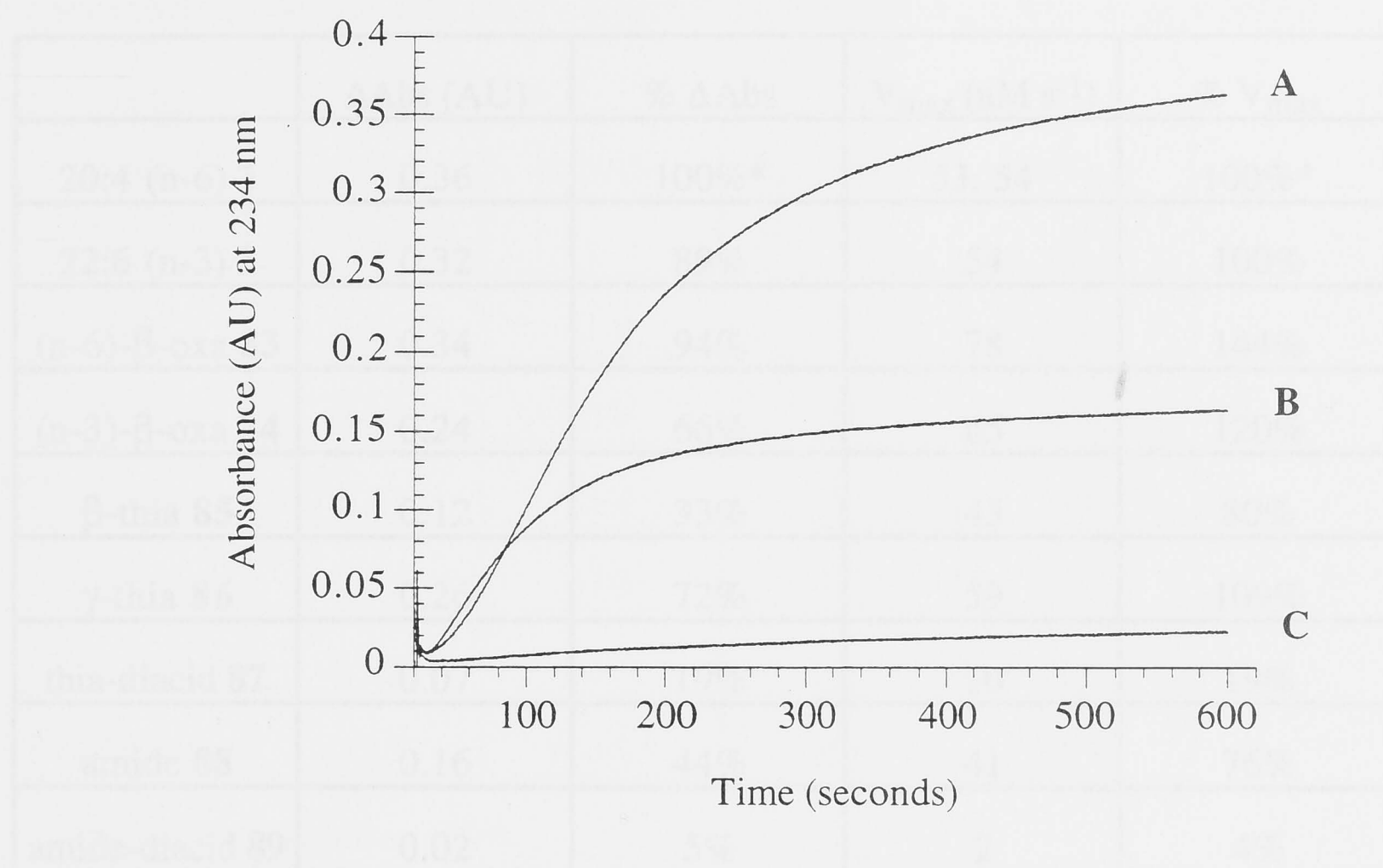
The experiments discussed above using the thio-ethers **85-87** indicate that soybean 15-LO tolerates the presence of the β -thia or γ -thia group in a substrate and readily oxidises (n-6) PUFAs of longer chain length than the 22 carbons present in docosahexaenoic acid **5**. In addition, 15-LO is able to oxidise an α -branched diacid although the rate of oxidation and yield of product are about one fifth the values recorded for arachidonic acid **3**.

Fig 4.4: Change in absorbance over time for reaction of the α -branched β -thia diacid **87** (**A** and **B**) with 15-LO concentrations of 1.6 and 0.8 $\mu\text{g} / \text{ml}$, respectively



The change in absorbance over time is shown in Fig 4.5 for arachidonic acid **3**, the amide **88** and the α -branched amide diacid **89** with soybean 15-LO (0.8 $\mu\text{g} / \text{ml}$). From Fig 4.5 it is evident that as the change in absorbance is greatest in the sample of arachidonic acid **3**, the naturally occurring substrate **3** reacts to a greater extent than the synthetic compounds **88** and **89**. The unbranched amide **88** reacts to about half the extent of arachidonic acid **3**. The lag time is shorter for the unbranched amide **88** than for arachidonic acid **3** implying that the ferrous ion at the active site of 15-LO is oxidised to form the active enzyme more rapidly with the synthetic substrate **88** than with arachidonic acid **3**. The maximum rate of change in absorbance which is a measure of the maximum rate of product formation is slower for the amide **88** than for arachidonic acid **3**. Like the results with the unbranched synthetic compounds **83-86** discussed earlier, after incubation with 15-LO for 5 minutes the rate of product formation from the amide **88** became slow relative to the rate of (15*S*)-HPETE (*S*)-**14** formation from arachidonic acid

Fig 4.5: Change in absorbance over time for reaction of the amide **88** (**B**), the α -branched amide diacid **89** (**C**) and arachidonic acid **3** (**A**) with 15-LO (0.8 $\mu\text{g} / \text{ml}$)



3. Hence, the amide **88** or the product formed by oxidation of the amide **88** inhibits soybean 15-LO more efficiently than the sample containing arachidonic acid **3** which continued to undergo oxidation for the 10 minute duration of the experiment.

During incubation with 15-LO the α -branched amide diacid **89** reacts very slowly as the change in absorbance is less than 10% of that observed for arachidonic acid **3**. To confirm that the apparent formation of conjugated diene from the α -branched amide diacid **89** was an enzyme catalysed reaction, the absorbance at 234 nm was monitored at two concentrations of 15-LO (0.8 and 1.6 $\mu\text{g} / \text{ml}$). Like the result shown in Fig 4.4 for the α -branched thio-ether diacid **87**, the total change in absorbance and rate of product formation from the diacid **89** approximately doubled when the enzyme concentration was doubled. Hence, the α -branched amide diacid **89** is a substrate for 15-LO although it is

Table 4.2: Absorbance change (ΔAbs (AU)) at 234 nm, the maximum rate of product formation (V_{max} (nM s⁻¹)), percentage of the absorbance change observed for arachidonic acid **3** (% ΔAbs) and percentage of the rate observed for arachidonic acid **3** (% V_{max}) during incubation of the substrates **3**, **5** and **83-89** with 15-LO (0.8 μg / ml)

	ΔAbs (AU)	% ΔAbs	V_{max} (nM s ⁻¹)	% V_{max}
20:4 (n-6) 3	0.36	100%*	53, 54	100%*
22:6 (n-3) 5	0.32	89%	54	100%
(n-6)- β -oxa 83	0.34	94%	78	144%
(n-3)- β -oxa 84	0.24	66%	65	120%
β -thia 85	0.12	33%	43	80%
γ -thia 86	0.26	72%	59	109%
thia-diacid 87	0.07	19%	10	19%
amide 88	0.16	44%	41	76%
amide-diacid 89	0.02	5%	2	4%

* Arbitrarily assigned as 100%

oxidised relatively slowly by the enzyme. From the above results, soybean 15-LO oxidises amino acid conjugates of (n-6) PUFAs.

Summarised in Table 4.2, soybean 15-LO has been shown to tolerate compounds with a range of functional groups as substrates. The relative rates of oxidation of PUFAs ranging in chain length from 16 to 22 carbons and unsaturation from diene to hexaene have been reported using soybean 15-LO.⁵³ All straight chain compounds acted as substrates including 17, 19 and 21-carbon compounds with the maximum rate of product formation for the 20, 21 and 22-carbon compounds greater than 60% of that observed for arachidonic acid **3**. The above results indicate that the presence of the amide, ether and thio-ether groups does not markedly change the relative maximum rates of LO catalysed oxidation compared to PUFAs of the same chain length without these functional groups. The α -branched compounds **87** and **89** are less suitable substrates for 15-LO than

Table 4.3: Absorbance change (ΔAbs (AU)) at 234 nm, maximum rate of product formation (V_{max} (nM s⁻¹)), percentage of the maximum absorbance change observed for arachidonic acid **3** (% ΔAbs) and percentage of the maximum rate observed for arachidonic acid **3** (% V_{max}) during incubation of the compound **3** with 5-LO at different concentrations of detergent (% v/v Tween[®] 20) and saturation with oxygen (+O₂)

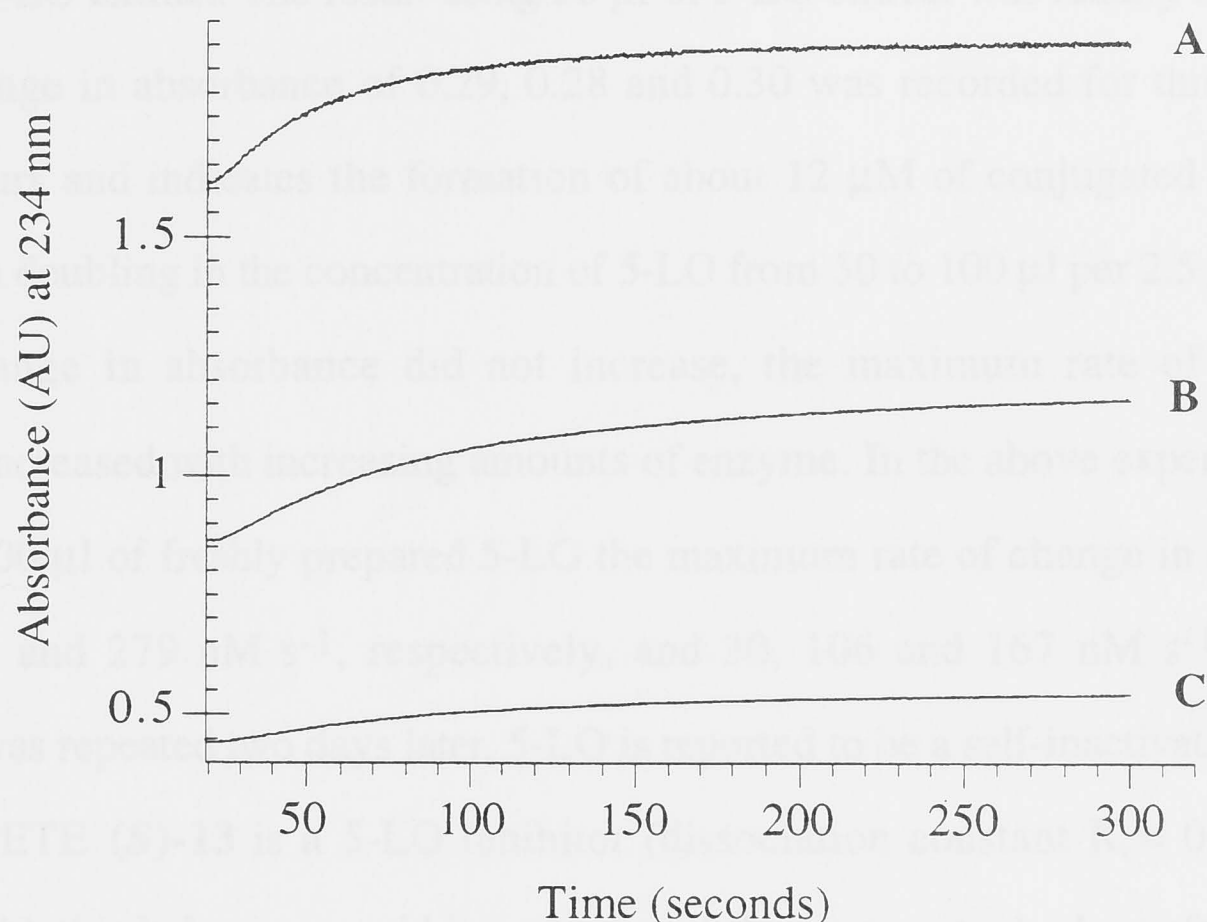
% v/v Tween [®] 20	ΔAbs	% ΔAbs	V_{max} (nM s ⁻¹)	% V_{max}
0%	0.16	61%	29	11%
0.0005%	0.17	65%	160	60%
0.005%	0.22	83%	155	58%
0.05%	0.11	41%	36	14%
0.5%	0.05	20%	11	4%
0.005% +O ₂	0.27	100%*	265	100%*

* Arbitrarily assigned as 100%

unbranched compounds. Hence, the part of the molecule containing the carboxylic acid group has an influence on binding to the enzyme active site despite the fact that enzyme catalysed oxidation occurs at the opposite end of the molecule.

With the intention of repeating the experiments outlined above with crude potato 5-LO extract instead of soybean 15-LO, a modification of the incubation conditions was investigated. As mentioned in Chapter 1, the use of a detergent improves the stability of 5-LO during preparation of the extract from potatoes.^{60,61} Using arachidonic acid **3** (50 μM) as the substrate for 5-LO, UV spectroscopy was used to demonstrate that a second detergent, Tween[®] 20, improves the yield of the conjugated *E,Z*-diene (**S**)-**14**. As summarised in Table 4.3, a concentration of 0.005% Tween[®] 20 by volume and saturation of the incubation buffer (0.1 M KH₂PO₄, pH = 6.0) with oxygen were found to increase the yield of product by about 60% based on the change in absorbance after 5 minutes. The maximum rate of product formation increased ninefold. The total change in

Fig 4.6: Change in absorbance over time observed for the reaction of arachidonic acid **3** (50 μ M) with crude 5-LO extract at 100 (A), 50 (B) and 25 μ l (C) per 2.5 ml solution



absorbance corresponds to a 15% yield of (5*S*)-HETE (**S**)-**14** after incubation of arachidonic acid **3** with potato 5-LO for 5 minutes under optimum conditions.

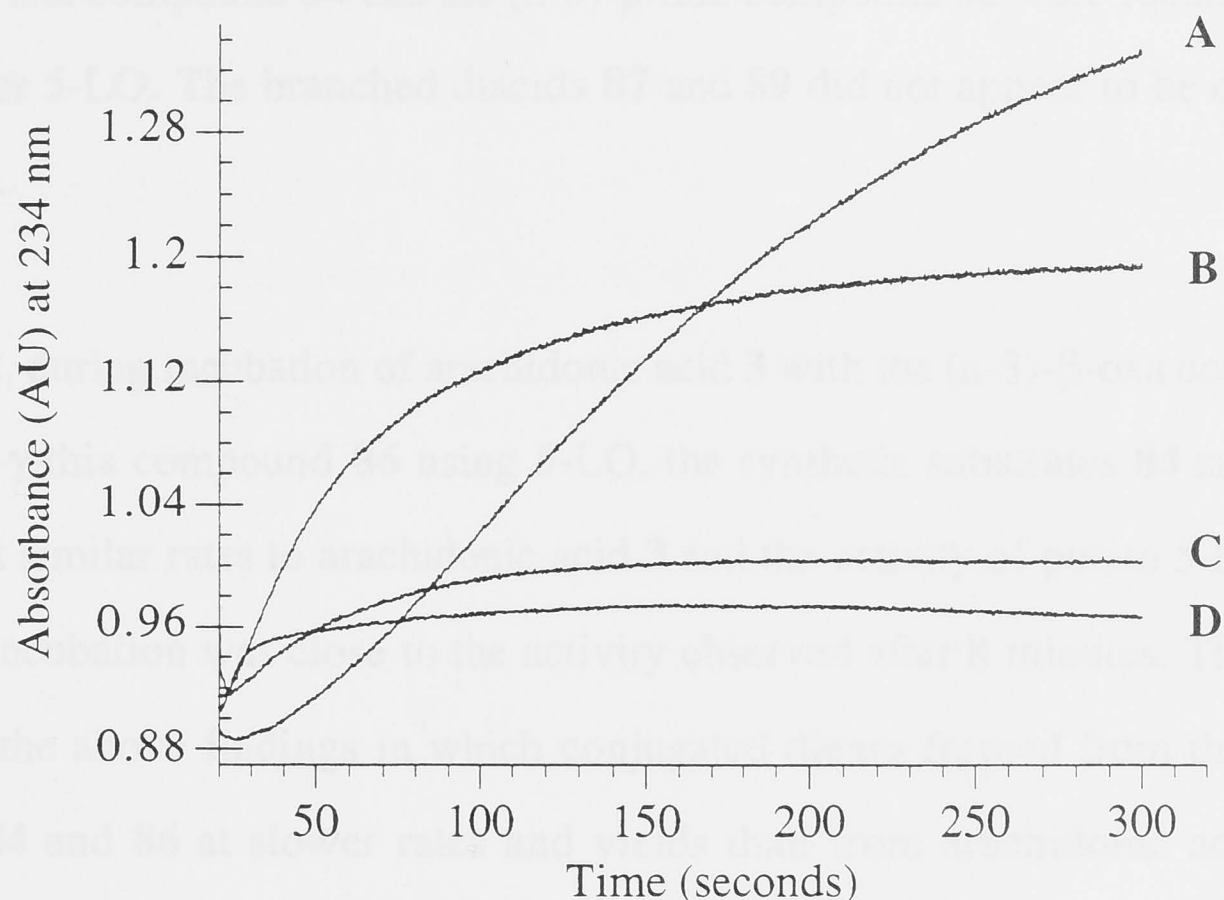
The turbidity of the crude potato 5-LO extract used in these experiments resulted in a high background absorbance, consequently reducing the signal to noise ratio and limiting the volume of extract used in the spectroscopic studies. Centrifugation slightly lowered the background absorbance and filtration destroyed the activity of the 5-LO extract. Further chromatography to purify the 5-LO protein has been reported to increase the activity of potato 5-LO but doubles the amount of time required to prepare 5-LO from potatoes to 2 days⁶⁰ without altering the short period (7 days) for which the purified extract retains LO activity.

For arachidonic acid **3** (50 μM) the volume of 5-LO extract used was 25, 50 and 100 μl in a total volume of 2.5 ml and as shown in Fig 4.6 the change in absorbance was 0.10, 0.29 and 0.30, respectively. The high background absorbance due to the turbidity of the crude enzyme extract altered the initial absorbance of samples containing different volumes of 5-LO extract. The result using 50 μl of 5-LO extract was readily reproduced, the total change in absorbance of 0.29, 0.28 and 0.30 was recorded for three samples over two hours and indicates the formation of about 12 μM of conjugated *E,Z*-diene. Whilst with a doubling in the concentration of 5-LO from 50 to 100 μl per 2.5 ml solution the total change in absorbance did not increase, the maximum rate of change in absorbance increased with increasing amounts of enzyme. In the above experiment with 25, 50 and 100 μl of freshly prepared 5-LO the maximum rate of change in absorbance was 92, 201 and 279 nM s^{-1} , respectively, and 30, 106 and 167 nM s^{-1} when the experiment was repeated two days later. 5-LO is reported to be a self-inactivating enzyme and (5*S*)-HPETE (*S*)-**13** is a 5-LO inhibitor (dissociation constant $K_i = 0.05 \mu\text{M}$).⁷⁶ Consistent with this behaviour rapid inactivation of the enzyme took place after less than 25% of the maximum theoretical change in absorbance had occurred.

At a concentration of 50 μM , the synthetic PUFAs **83-89** were monitored for 5 minutes under the same conditions as arachidonic acid **3** with 50 μl of 5-LO extract per 2.5 ml sample. The spectroscopic study of each compound with potato 5-LO was repeated two weeks after the initial experiment with a second preparation of 5-LO to assess the reproducibility of the results. In Fig 4.7 the change in absorbance over time is shown for the incubation of arachidonic acid **3**, the β -oxa fatty acid **83**, the γ -thia fatty acid **86** and the amide **88** with potato 5-LO.

As a change in absorbance is observed over time in each case in Fig 4.7, each of the PUFAs **3**, **83**, **86** and **88** is a substrate for 5-LO. The amide **88** is oxidised to a conjugated diene at about one third of the rate of arachidonic acid **3** oxidation. The decline in 5-LO activity observed within the first two minutes during incubation with arachidonic acid **3** is relatively small for the amide **88** and only begins to take effect after four minutes

Fig 4.7: Change in absorbance over time for reaction of the unbranched amide **88** (A), arachidonic acid **3** (B), the γ -thia fatty acid **86** (C) and the (n-6)- β -oxa fatty acid **83** (D) with 5-LO



of incubation. Consequently, the total change in absorbance observed after incubation for 5 minutes with the amide **88** is greater than that observed with arachidonic acid **3**. The β -oxa compound **83** and the γ -thia compound **86** are poor substrates for 5-LO as the amount of diene formed from the synthetic compounds **83** and **86** is less than 40% of the amount of diene formed from arachidonic acid **3**. After the maximum rate of product formation was reached for the compounds **83** and **86** within the first minute of incubation, a decline in reaction rate was observed similar to the decline observed using arachidonic acid **3** as the substrate. However, with the (n-6)- β -oxa compound **83**, the maximum rate was close to the value recorded for arachidonic acid **3** but an abrupt decline in reaction rate was observed after less than 40 seconds with 5-LO. The lack of a marked decline in enzyme activity during incubation of 5-LO with the amide **88** and the high initial enzyme activity followed by a sudden decline in activity with the (n-6)- β -oxa fatty

acid **83** were both reproducible results. More detailed study of these unusual substrate effects was not undertaken because of the relatively poor 5-LO activity observed with the substrate **83**, the instability of 5-LO and the high background absorbance of the 5-LO extract.

The (n-3)- β -oxa compound **84** and the (n-6)- β -thia compound **85** were found to be poor substrates for 5-LO. The branched diacids **87** and **89** did not appear to be oxidised by potato 5-LO.

In Chapter 2, during incubation of arachidonic acid **3** with the (n-3)- β -oxa compound **84** or the (n-3)- γ -thia compound **86** using 5-LO, the synthetic substrates **84** and **86** were consumed at similar rates to arachidonic acid **3** and the activity of potato 5-LO after 16 minutes of incubation was close to the activity observed after 8 minutes. These results differ from the above findings in which conjugated dienes formed from the synthetic substrates **84** and **86** at slower rates and yields than from arachidonic acid **3** and a decline in 5-LO activity occurred within the first two minutes of incubation. However, the results of both studies imply that 5-LO catalyses the oxidation of the synthetic compounds **84** and **86** and only identification of the oxidation products would provide a means of confirming this beyond dispute.

The spectroscopic study of product formation from the synthetic compounds **83-89** using 5-LO indicates that during incubation with potato 5-LO products containing the conjugated diene moiety are formed from PUFAs containing β -oxa, β -thia, γ -thia and amide functional groups but compounds containing the α -branched β -thia and α -branched amide groups are not oxidised by 5-LO. Apart from the amide **88**, the rates of reaction and yields of conjugated diene are low relative to arachidonic acid **3**.

In conclusion, UV spectroscopy has been used to confirm that conjugated dienes form during the incubation of the straight chain PUFAs **83-86** and **88** with both soybean 15-LO and potato 5-LO. In addition conjugated dienes form during the incubation of the α -

branched diacids **87** and **89** with 15-LO but diene formation does not occur using potato 5-LO. Based on the change in absorbance at 234 nm, the yields of products from the synthetic compounds **83-89** are lower than the yields of the HPETE isomers **12-14** and **44-46** from arachidonic acid **3** using 15-LO or 5-LO. Based on the yields of diene in each case, soybean 15-LO tolerates the synthetic compounds **83-89** as substrates more readily than potato 5-LO which in general forms products from the synthetic compounds **83-86** and **88** at a slow rate and low yield relative to arachidonic acid **3**. The formation of conjugated dienes over time with 15-LO and 5-LO is a property consistent with enzyme catalysed oxidation of the synthetic PUFAs **83-89** to form hydroperoxides.

Product studies of the interaction of modified fatty acids with lipoxygenases

In Chapter 2, an HPLC assay of fatty acid mixtures was used to establish that LO enzymes catalysed the consumption of arachidonic acid **3** and the synthetic PUFAs **83**, **84** and **86**. In Chapter 3, UV spectroscopy was used to confirm that the oxidation of the individual synthetic PUFAs **83-89** was catalysed by soybean 15-LO. In addition by UV spectroscopy it was evident that at least several of the synthetic PUFAs **83-89** were also oxidised by potato 5-LO. The aim of the work presented in this Chapter was to isolate and identify each of the products formed by LO catalysed oxidation of the synthetic PUFAs **83-89** and to assess the regioselectivity of each LO catalysed oxidation by the unambiguous assignment of structure to each product.

In Chapter 1 techniques were developed to isolate and identify (15*S*)-HETE (*S*)-**53** which was formed by reducing the hydroperoxide product (*S*)-**14** of soybean 15-LO catalysed arachidonic acid **3** oxidation. These techniques are applicable to the isolation of the oxidation products formed from each of the synthetic PUFAs **83-89** and the assignment of structure to each product. Hence, the (n-6)- β -oxa compound **83** (50 mg) was oxidised by incubation with 15-LO (15 mg) in buffer (0.1 M KH_2PO_4 , pH = 9.0, 200 ml) at 30 °C for 30 minutes with oxygen bubbling through the solution. Triphenylphosphine (50 mg) dissolved in dichloromethane (200 ml) and hydrochloric acid (0.2 N, 20 ml) were added to the solution which was then stirred at 0 °C for 45 minutes. The product was then extracted from the aqueous layer with dichloromethane (2 x 200 ml). A sample of the crude product mixture was analysed by HPLC with detection of the conjugated diene moiety at 234 nm as shown in Fig 5.1. A single conjugated diene appears to be the main product as one major peak was observed with a retention time of 15 minutes. The solvent was evaporated from the combined organic extracts and the residue was purified by flash chromatography (ethyl acetate : hexane : acetic acid, 80 : 20 : 1) to yield the product (12 mg). As a low mass recovery was observed during flash chromatography and semi-preparative HPLC of oxidation products, samples were

Fig 5.1: HPLC of the products derived from oxidation of the (n-6)- β -oxa compound **83** using 15-LO

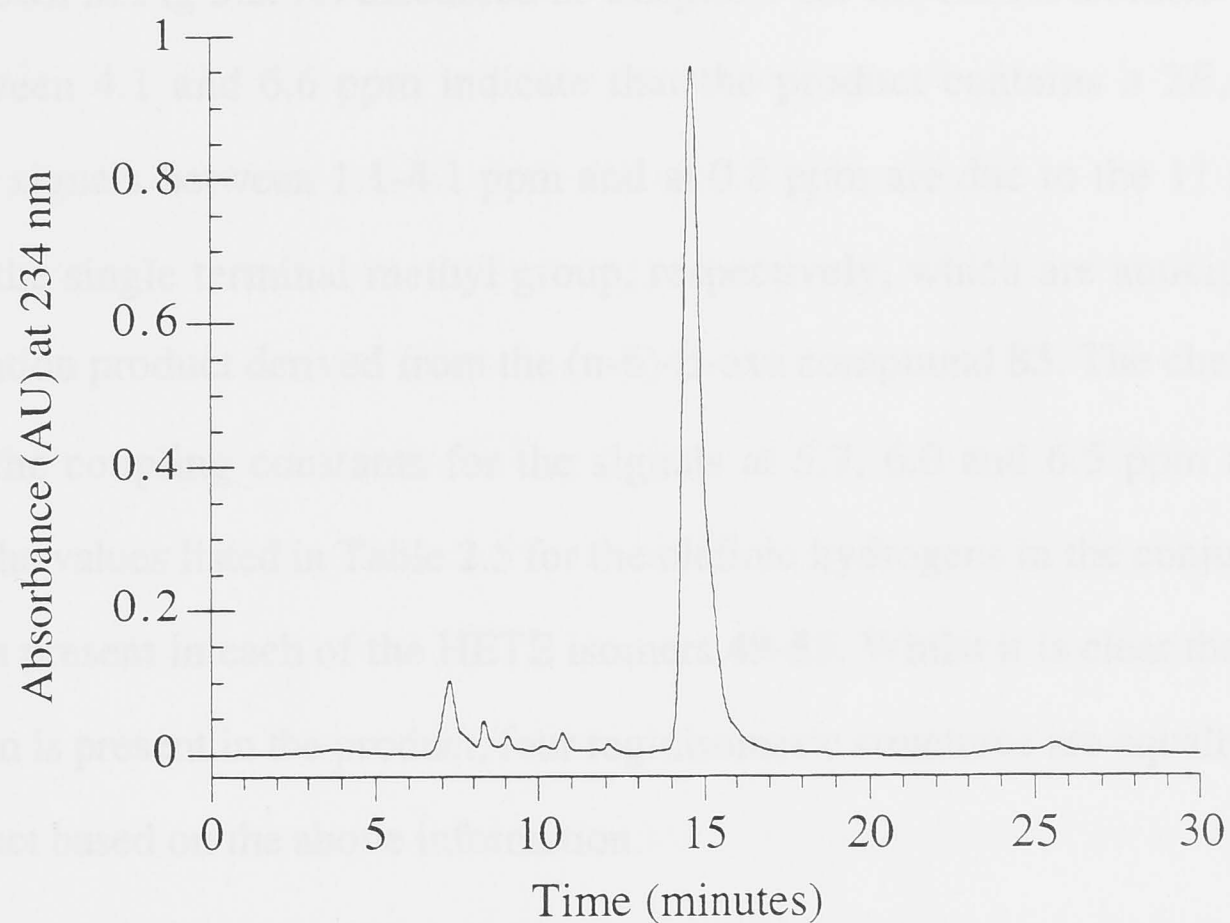
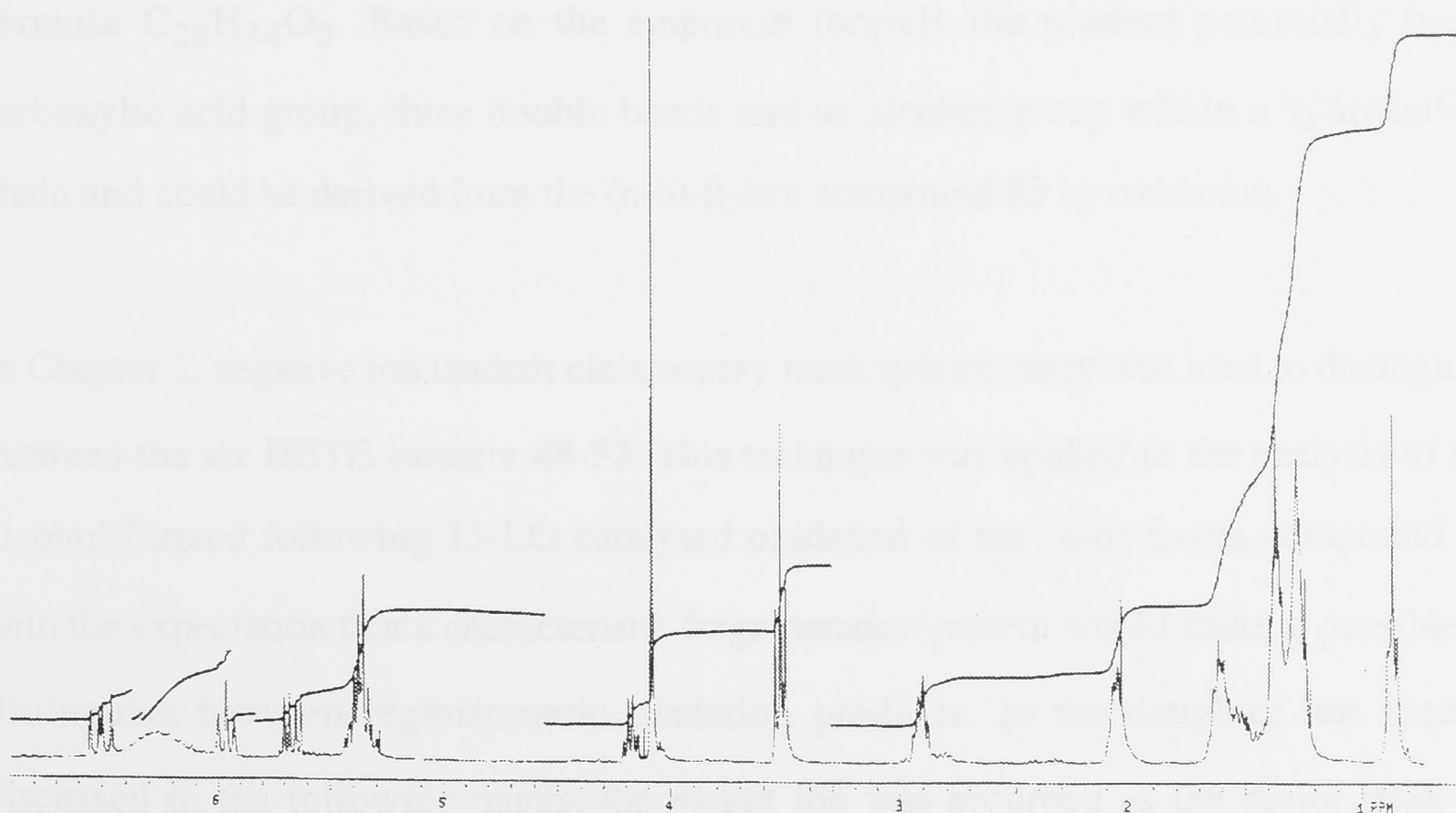


Fig 5.2: ^1H nmr spectrum of the main product derived from the oxidation of the (n-6)- β -oxa compound **83** using 15-LO



purified by flash chromatography with a lower than normal quantity of silica to minimise decomposition.

The ^1H nmr spectrum for the product formed from the (n-6)- β -oxa compound **83** using 15-LO is shown in Fig 5.2. As discussed in Chapter 1 for the HETE isomers **49-53**, the signals between 4.1 and 6.6 ppm indicate that the product contains a 2*E*,4*Z*-dienol system. The signals between 1.1-4.1 ppm and at 0.8 ppm are due to the 11 methylene groups and the single terminal methyl group, respectively, which are anticipated for a simple oxidation product derived from the (n-6)- β -oxa compound **83**. The chemical shift values and the coupling constants for the signals at 5.7, 6.0 and 6.5 ppm are almost identical to the values listed in Table 2.5 for the olefinic hydrogens in the conjugated *E,Z*-diene system present in each of the HETE isomers **49-53**. Whilst it is clear that a 2*E*,4*Z*-dienol system is present in the product, four regioisomeric structures are equally plausible for the product based on the above information.

The accurate mass for the sodium adduct of the product was determined by positive ion electrospray mass spectrometry. The accurate mass was observed to be 361.2361 and based on a calculated mass of 361.2355 the empirical formula of the sodium adduct is $\text{C}_{20}\text{H}_{34}\text{O}_4\text{Na}^+$. Hence, the empirical formula of the reaction product is $\text{C}_{20}\text{H}_{34}\text{O}_4$ and the product contains one more oxygen than the (n-6)- β -oxa compound **83** which has the formula $\text{C}_{20}\text{H}_{34}\text{O}_3$. Based on the empirical formula the product potentially has a carboxylic acid group, three double bonds and an alcohol group within a hydrocarbon chain and could be derived from the (n-6)- β -oxa compound **83** by oxidation.

In Chapter 1, negative ion tandem electrospray mass spectrometry was used to distinguish between the six HETE isomers **48-53**. This technique was applied to the analysis of the alcohol formed following 15-LO catalysed oxidation of the (n-6)- β -oxa compound **83** with the expectation that a characteristic fragmentation pattern would make it possible to distinguish between regioisomeric oxidation products. In the daughter ion spectra discussed in the following pages, the parent ion was recorded as the major peak by

negative ion electrospray mass spectrometry and the daughter ions were formed by collision induced dissociation (CID) with argon.

The mass spectrum for the product formed by oxidation of the (n-6)- β -oxa compound **83** and the structure assigned to the product **96** are shown in Fig 5.3. A molecular anion was observed with m/z 337, the ion at m/z 319 corresponds to the loss of H_2O from the molecular anion and the ion at m/z 75 corresponds to $[OCH_2CO_2H]^-$ which results from a break in the covalent bond between the β -oxygen and C-1'. The ion at m/z 237 can be used to assign the location of the alcohol group to C-13' and results from the loss of the neutral fragment with the formula $C_6H_{12}O$. Loss of the same neutral fragment was recorded in the daughter ion spectrum for 15-HETE **53**. Based on the types of daughter ions observed to form from the HETE isomers **48-53** shown in Scheme 2.4, the daughter ion at m/z 237 can only be derived from the molecule **96** with the structure shown and cannot form from any of the other three possible structures for dienol reaction products derived from the oxidation of the (n-6)- β -oxa compound **83**. So like the 15-LO catalysed oxidation of arachidonic acid **3**, oxidation of the (n-6)- β -oxa compound **83** by 15-LO occurs at the n-6 carbon.

By following a similar procedure to that outlined above for the (n-6)- β -oxa compound **83**, a single product was isolated following 15-LO catalysed oxidation of each of the synthetic compounds **83-89** followed by reduction with sodium borohydride. By HPLC a single major product containing a conjugated diene system was observed in each case. By 1H nmr spectroscopy as summarised in Table 5.1, the chemical shift values and coupling constants for the olefinic hydrogens between 5.5 and 6.6 ppm and the signal between 4.1 and 4.2 ppm corresponded with the values reported earlier for each of the HETE isomers **49-53** and are consistent with the presence of a 2*E*,4*Z*-dienol system in each product. Products of 15-LO catalysed oxidation of the synthetic compounds **84**, **85**, **87** and **88** were analysed by ^{13}C nmr spectroscopy and characteristic signals were observed for the olefinic carbons between 125 and 137 ppm and between 72 and 74 ppm for the carbon bonded to the alcohol group. So a single major product was observed

Fig 5.3: The electrospray MS/MS spectrum for the product **96** from the oxidation of the (n-6)- β -oxa compound **83** by 15-LO

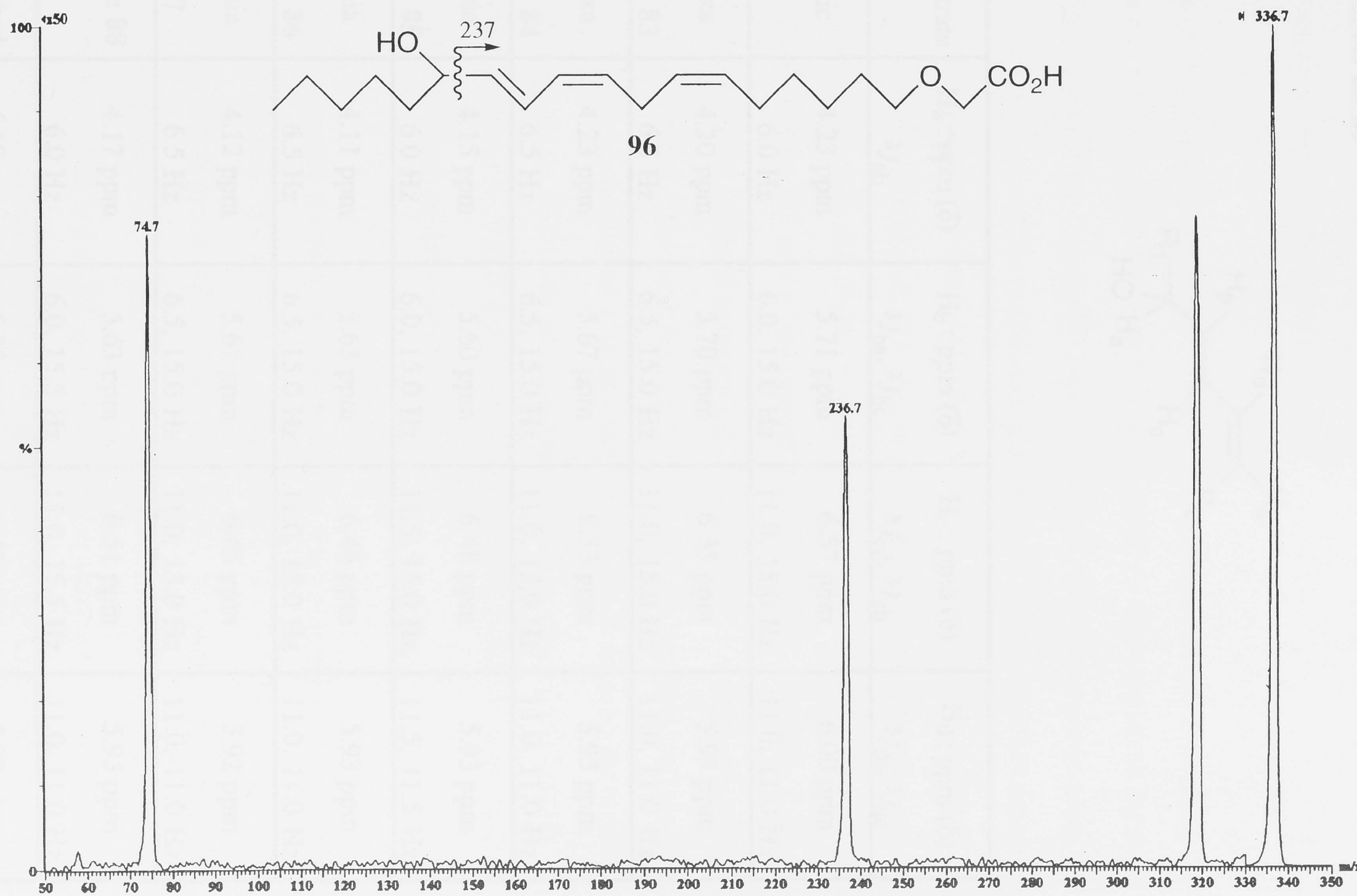
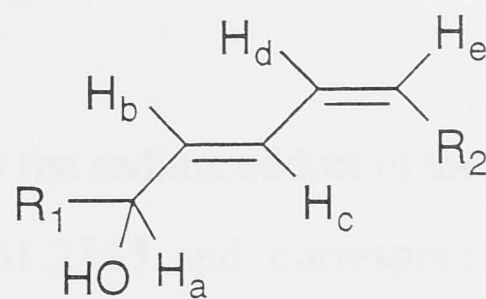


Table 5.1: Chemical shift values and vicinal coupling constants (3J) in the ^1H nmr spectra of products derived from 15-LO catalysed oxidation of arachidonic acid **3** and the synthetic PUFAs **83-89**



15-LO substrate	H_a : ppm (δ) $^3J_{ab}$	H_b : ppm (δ) $^3J_{ba}, ^3J_{bc}$	H_c : ppm (δ) $^3J_{cd}, ^3J_{cb}$	H_d : ppm (δ) $^3J_{dc}, ^3J_{de}$
arachidonic acid 3	4.23 ppm 6.0 Hz	5.71 ppm 6.0, 15.0 Hz	6.57 ppm 11.0, 15.0 Hz	6.00 ppm 11.0, 11.0 Hz
(n-6)- β -oxa compound 83	4.20 ppm 6.5 Hz	5.70 ppm 6.5, 15.0 Hz	6.55 ppm 11.0, 15.0 Hz	5.99 ppm 11.0, 11.0 Hz
(n-3)- β -oxa compound 84	4.23 ppm 6.5 Hz	5.67 ppm 6.5, 15.0 Hz	6.53 ppm 11.0, 15.0 Hz	5.95 ppm 11.0, 11.0 Hz
(n-6)- β -thia compound 85	4.15 ppm 6.0 Hz	5.60 ppm 6.0, 15.0 Hz	6.48 ppm 11.5, 15.0 Hz	5.93 ppm 11.5, 11.5 Hz
(n-6)- γ -thia compound 86	4.11 ppm 6.5 Hz	5.63 ppm 6.5, 15.0 Hz	6.46 ppm 11.0, 15.0 Hz	5.93 ppm 11.0, 11.0 Hz
(n-6)- β -thia diacid 87	4.12 ppm 6.5 Hz	5.61 ppm 6.5, 15.0 Hz	6.45 ppm 11.0, 15.0 Hz	5.92 ppm 11.0, 11.0 Hz
(n-6)-amide 88	4.17 ppm 6.0 Hz	5.63 ppm 6.0, 15.5 Hz	6.51 ppm 11.0, 15.5 Hz	5.93 ppm 11.0, 11.0 Hz
(n-6)-branched amide 89	4.18 ppm 6.5 Hz	5.56 ppm 6.5, 15.0 Hz	6.49 ppm 11.0, 15.0 Hz	5.93 ppm 11.0, 11.0 Hz

following 15-LO catalysed oxidation of the synthetic PUFAs **83-89** and a 2*E*,4*Z*-dienol system was present in each product. As the location of the hydroxyl group could not be determined by nmr spectroscopy, negative ion tandem electrospray mass spectrometric analysis of each compound was performed.

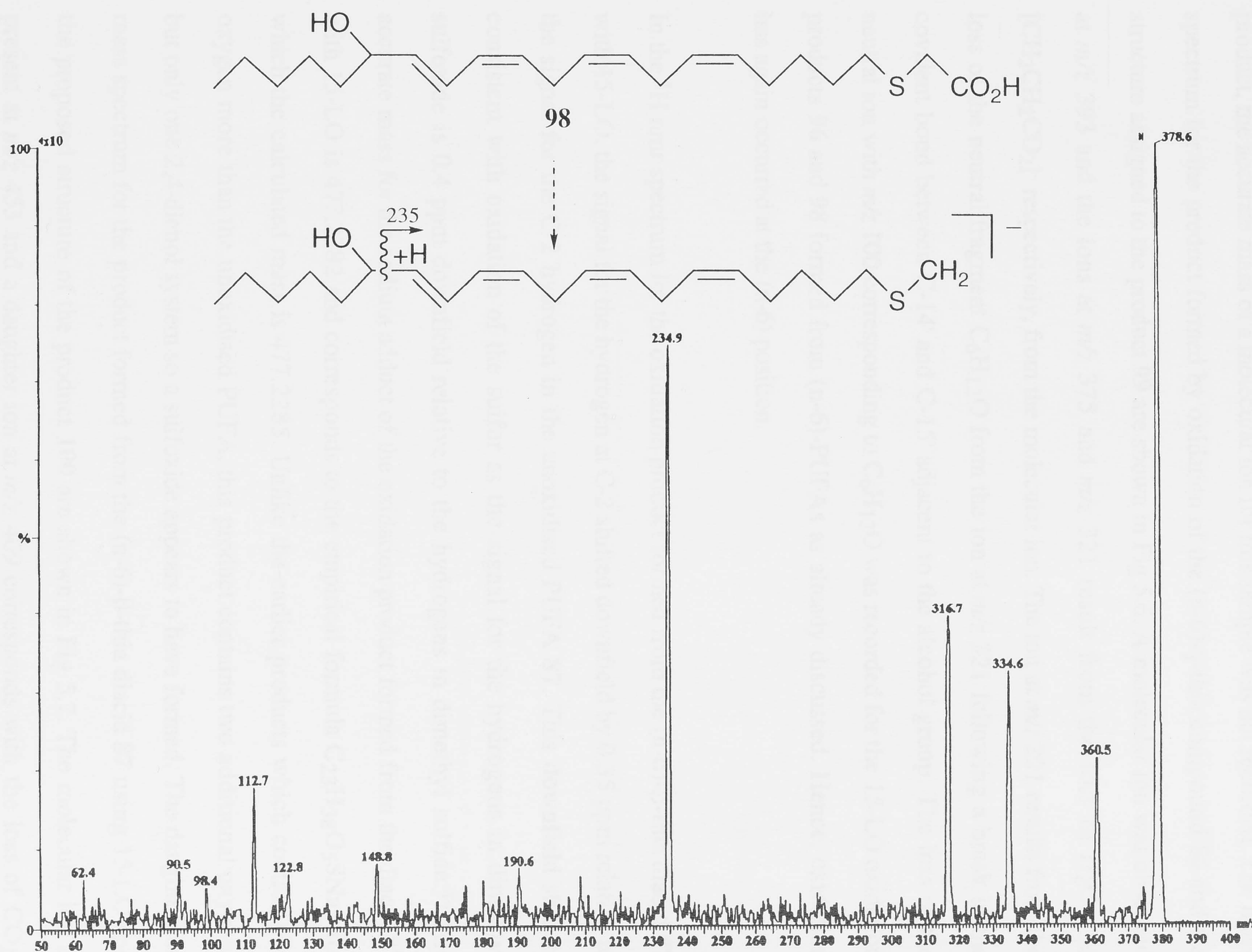
The accurate mass recorded for the sodium adduct of the product formed from the (n-3)- β -oxa compound **84** was 361.2345 and corresponded to the empirical formula $C_{20}H_{34}O_4Na^+$ for which the calculated mass is 361.2355. The mass spectrum for the product formed by oxidation of the (n-3)- β -oxa compound **84** and the structure assigned to the product **97** are shown in Fig 5.4. Similar to the result in Fig 5.3, in Fig 5.4 a molecular anion is observed with m/z 337, the ion at m/z 319 corresponds to the loss of H_2O from the molecular anion and the ion at m/z 75 corresponds to $[OCH_2CO_2H]^-$. The ion at m/z 239 results from the loss of the neutral fragment with the formula $C_6H_{10}O$ which forms *via* a break in the bond between C-12' and C-13'. The ions at m/z 267 and m/z 268 result from a break in the bond between C-13' and C-14'. The hydroxyl group is located at C-13' because from Chapter 1 daughter ions are known to form by the breaking of carbon-carbon bonds on either side of the carbon bonded to the hydroxyl group. So like the naturally occurring (n-3) PUFA docosahexaenoic acid **5**, oxidation of the (n-3)- β -oxa compound **84** occurs at the (n-6) position.

The accurate mass recorded for the sodium adduct of the product formed from the (n-6)- β -thia compound **85** was 403.2290 and corresponded to the empirical formula $C_{22}H_{36}O_3SNa^+$ for which the calculated mass is 403.2283. The mass spectrum for the product formed by oxidation of the (n-6)- β -thia compound **85** and the structure assigned to the product **98** are shown in Fig 5.5. A molecular ion is observed at m/z 379 and the ions at m/z 361, m/z 335 and m/z 317 result from the loss of H_2O , CO_2 and both H_2O and CO_2 , respectively, from the molecular ion. The ion at m/z 235 results from the loss of the neutral fragment $C_6H_{12}O$ from the ion at m/z 335 following a break in the covalent bond between C-14' and C-15' adjacent to the alcohol group. Hence, oxidation has again occurred at the (n-6) position.

Fig 5.4: The electrospray MS/MS spectrum for the product **97** from the oxidation of the (n-3)- β -oxa compound **84** by 15-LO



Fig 5.5: The electrospray MS/MS spectrum for the product **98** from the oxidation of the (n-6)- β -thia compound **85** by 15-LO



The compound formed from 15-LO catalysed oxidation of the (n-6)- γ -thia compound **86** decomposed following storage at $-30\text{ }^{\circ}\text{C}$ for 3 months. As a result of the instability of the product, the accurate mass of a molecular ion for this sample was not recorded. The mass spectrum for the product formed by oxidation of the (n-6)- γ -thia compound **86** and the structure assigned to the product **99** are shown in Fig 5.6. A molecular ion was observed at m/z 393 and the ions at m/z 375 and m/z 321 result from the loss of H_2O and $[\text{CH}_2\text{CH}_2\text{CO}_2]^-$ respectively, from the molecular ion. The ion at m/z 221 results from the loss of the neutral fragment $\text{C}_6\text{H}_{12}\text{O}$ from the ion at m/z 321 following a break in the covalent bond between C-14' and C-15' adjacent to the alcohol group. The loss of the neutral ion with m/z 100 corresponding to $\text{C}_6\text{H}_{12}\text{O}$ was recorded for the 15-LO oxidation products **96** and **98** formed from (n-6)-PUFAs as already discussed. Hence, oxidation has again occurred at the (n-6) position.

In the ^1H nmr spectrum for the oxidation product formed from the (n-6)- β -thia diacid **87** with 15-LO, the signal for the hydrogen at C-2 shifted downfield by 0.35 ppm relative to the signal for the C-2 hydrogen in the unoxidised PUFA **87**. This downfield shift is consistent with oxidation of the sulfur as the signal for the hydrogens in dimethyl sulfoxide is 0.4 ppm downfield relative to the hydrogens in dimethyl sulfide.⁴⁴ The accurate mass for the sodium adduct of the oxidation product formed from the diacid **87** with 15-LO is 477.2282 and corresponds to the empirical formula $\text{C}_{23}\text{H}_{38}\text{O}_5\text{SNa}^+$ for which the calculated mass is 477.2285. Unlike the earlier products which contain one oxygen more than the unoxidised PUFA, this product contains two additional oxygens but only one 2,4-dienol system so a sulfoxide appears to have formed. The daughter ion mass spectrum for the product formed from the (n-6)- β -thia diacid **87** using 15-LO and the proposed structure of the product **100** are shown in Fig 5.7. The molecular ion is present at m/z 453 and a daughter ion at m/z 409 corresponds with the loss of CO_2 . Consistent with the loss of the neutral ion $\text{C}_6\text{H}_{12}\text{O}$ with m/z 100 observed for the earlier products from oxidation with 15-LO, the ions at m/z 219 and m/z 173 may result from the loss of this fragment from the ions at m/z 319 and m/z 273, respectively. The ion at m/z 319 may form following the loss of $[\text{HO}_2\text{CCHCHCO}_2\text{H}]$ and H_2O from the molecular

Fig 5.6: The electrospray MS/MS spectrum for the product **99** from the oxidation of the (n-6)- γ -thia compound **86** by 15-LO

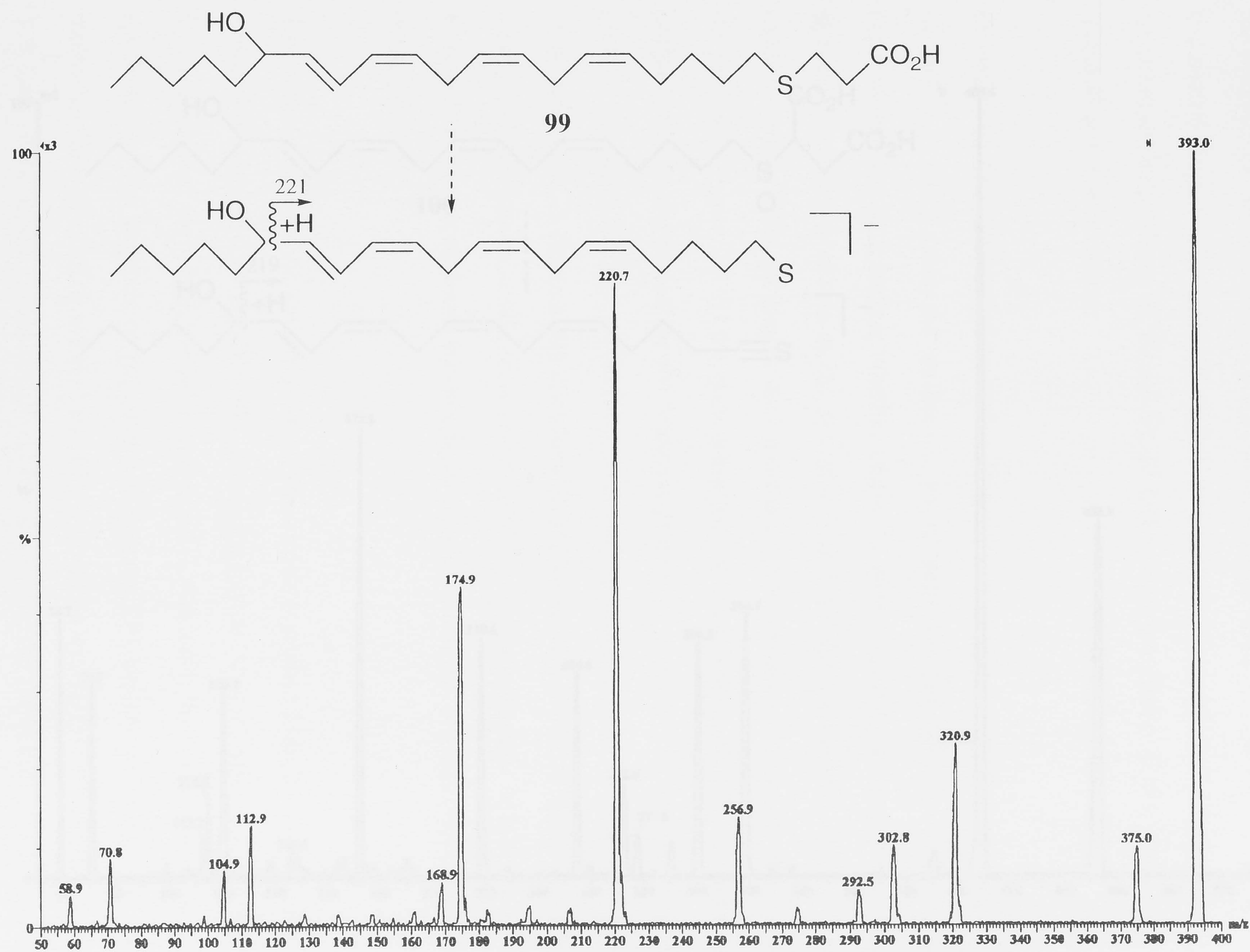
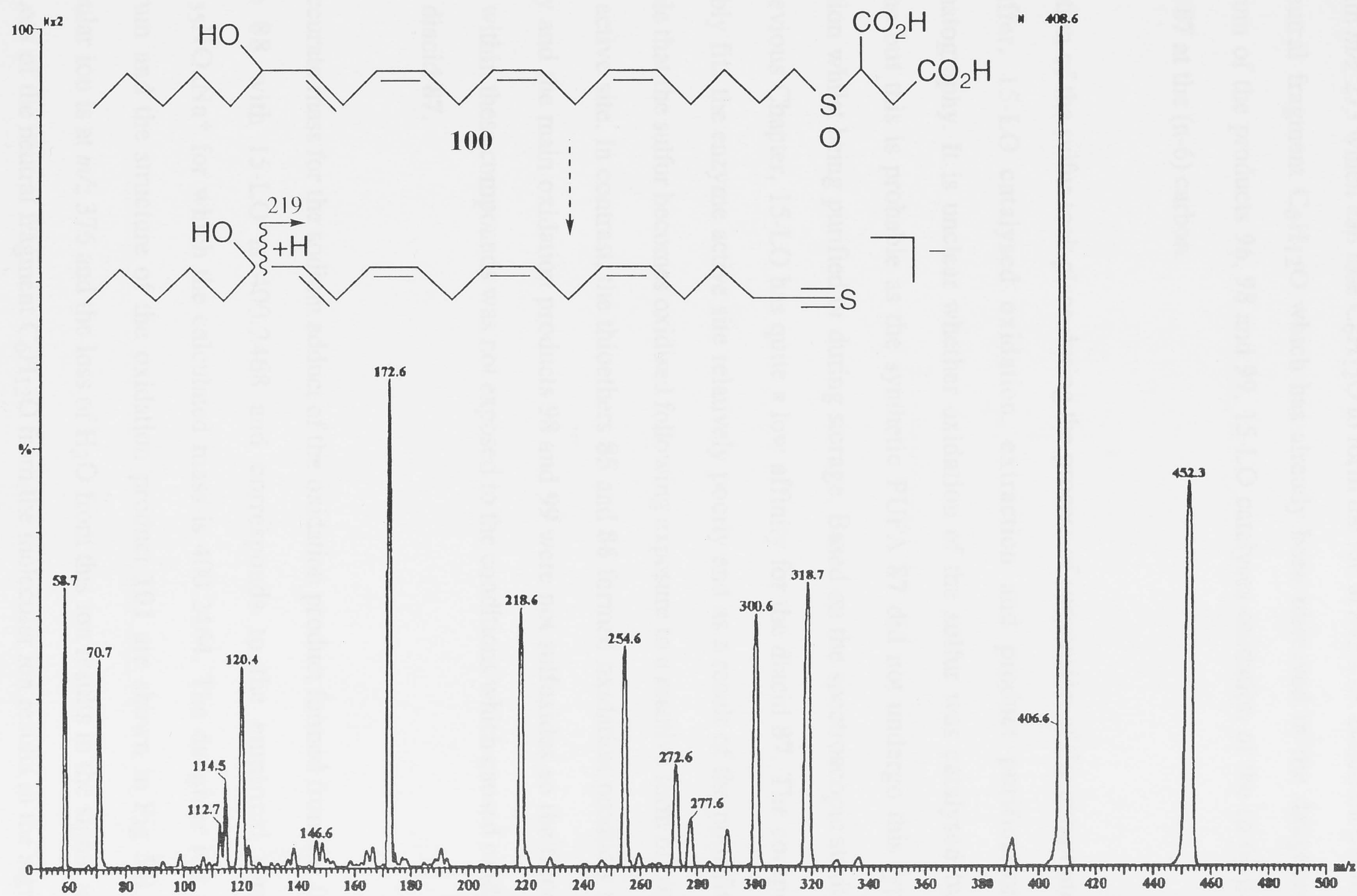


Fig 5.7: The electrospray MS/MS spectrum for the product **100** from the oxidation of the α -branched (n-6)- β -thia diacid **87** by 15-LO

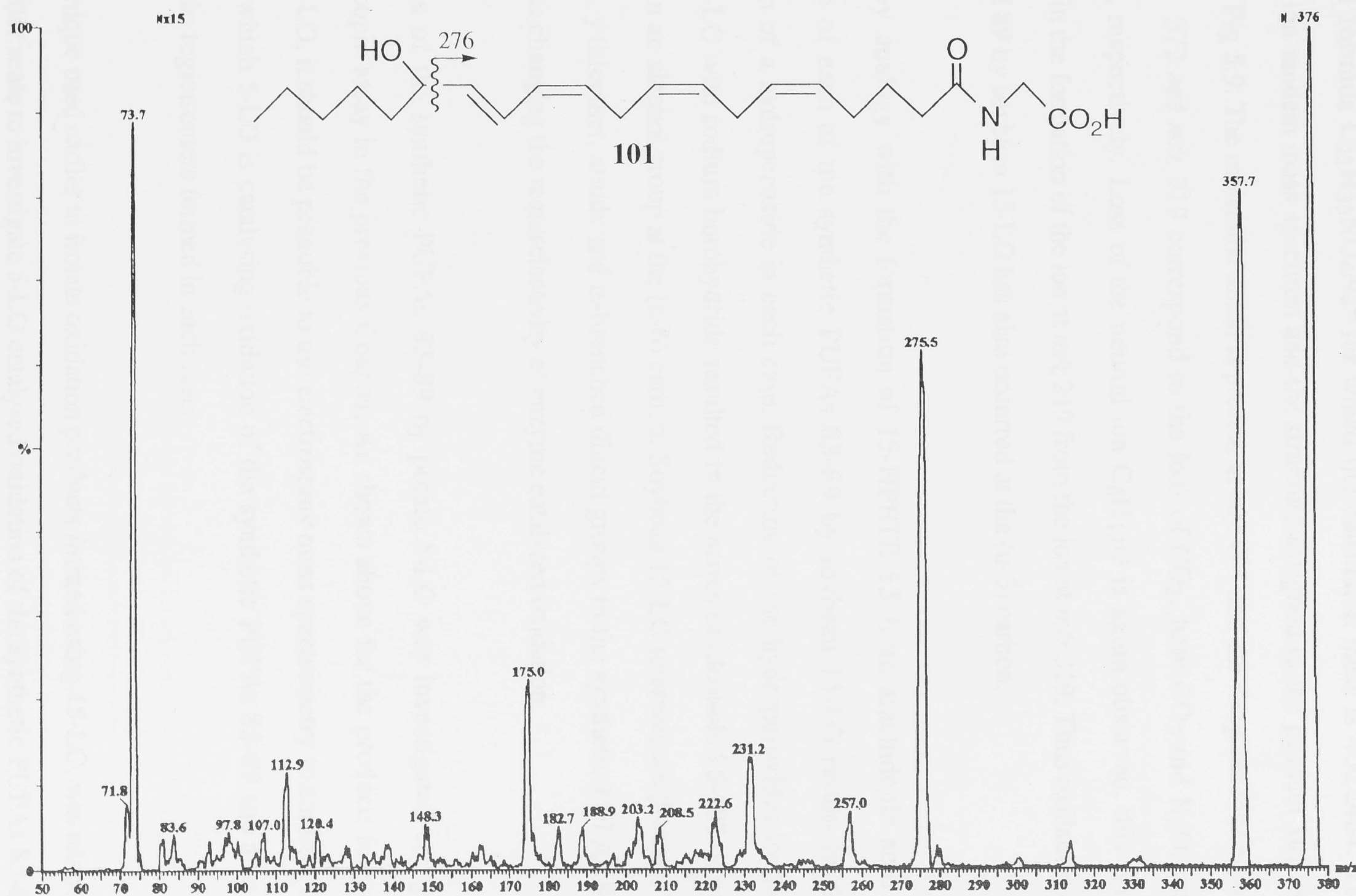


ion. This ion loses the fragment with m/z 100 and is also able to lose H_2O to form the ion with m/z 301. Loss of the neutral fragment [HCSH] from the ion at m/z 319 leads to the ion with m/z 273 which can lose $C_6H_{12}O$ to form the ion at m/z 173. Based on the loss of the neutral fragment $C_6H_{12}O$ which has already been observed in the daughter ion spectrum of the products **96**, **98** and **99**, 15-LO catalyses oxidation of the (n-6)- β -thia diacid **87** at the (n-6) carbon.

Oxidation of the sulfur took place during the process of suspension of the compound **87** in buffer, 15-LO catalysed oxidation, extraction and product purification by chromatography. It is unclear whether oxidation of the sulfur was catalysed by the enzyme but this is probable as the synthetic PUFA **87** did not undergo this type of oxidation whilst being purified or during storage. Based on the spectroscopic studies in the previous Chapter, 15-LO has quite a low affinity for the diacid **87**. The compound probably fits the enzyme active site relatively poorly and as a result of the poor fit it is possible that the sulfur becomes oxidised following exposure to a reactive form of oxygen at the active site. In contrast, the thioethers **85** and **86** formed oxidation products more readily and the main oxidation products **98** and **99** were not sulfoxides so the thioether group within these compounds was not exposed to the conditions which caused oxidation of the diacid **87**.

The accurate mass for the sodium adduct of the oxidation product formed from the (n-6)-amide **88** with 15-LO is 400.2468 and corresponds to the empirical formula $C_{22}H_{35}NO_4Na^+$ for which the calculated mass is 400.2464. The daughter ion mass spectrum and the structure of the oxidation product **101** are shown in Fig 5.8. The molecular ion is at m/z 376 and the loss of H_2O from this ion results in the signal at m/z 358. Loss of the neutral fragment $C_6H_{12}O$ from the molecular ion results in the signal at m/z 276 and the loss of CO_2H and both CO_2 and $C(O)NHCH_2$ from this ion results in the peaks at m/z 231 and m/z 175, respectively. Loss of the same neutral fragment with m/z 100 was observed in the earlier cases so the product **101** also has the alcohol group attached to the (n-6) carbon.

Fig 5.8: The electrospray MS/MS spectrum for the product **101** from the oxidation of the (n-6)-amide **88** by 15-LO



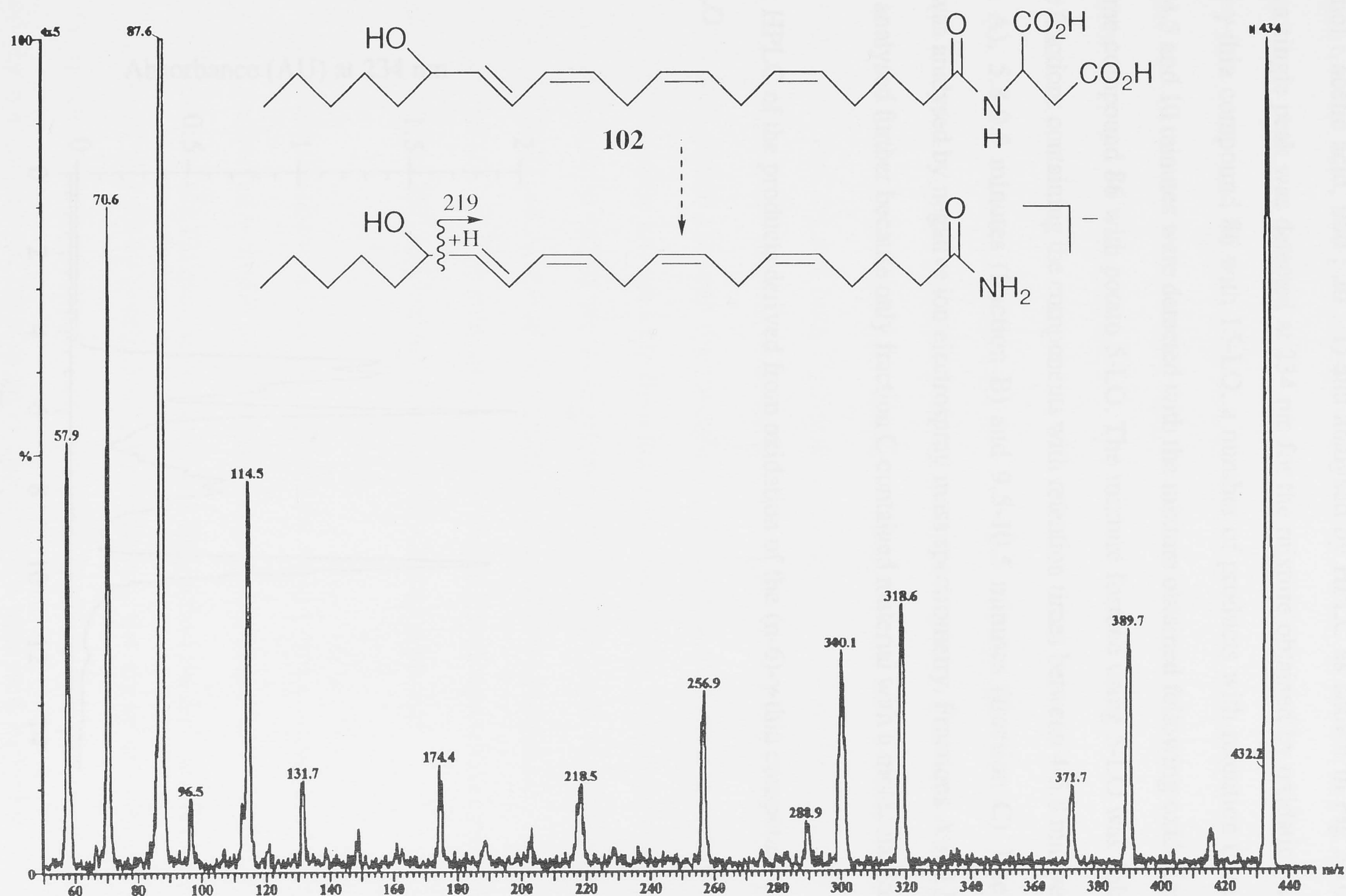
The final 15-LO oxidation product was derived from the α -branched amide **89** and the accurate mass of the sodium adduct for this product is 400.2452 and corresponds to the empirical formula $C_{22}H_{35}NO_4Na^+$ for which the calculated mass is 400.2464. The negative ion tandem mass spectrum and the structure assigned to the product **102** are shown in Fig 5.9. The molecular anion is present at m/z 434 and the daughter ions at m/z 390, m/z 372 and m/z 319 correspond to the loss of CO_2 , both CO_2 and H_2O , and $C_4H_3O_4$, respectively. Loss of the neutral ion $C_6H_{12}O$ is again observed, this time resulting in the formation of the ion at m/z 219 from the ion at m/z 319. Thus oxidation of the diacid **89** by soybean 15-LO has also occurred at the (n-6) carbon.

Hence, by analogy with the formation of 15-HPETE **53** from arachidonic acid **3** oxidation of each of the synthetic PUFAs **83-89** by soybean 15-LO results in the formation of a hydroperoxide in each case. Reduction of the hydroperoxides formed using 15-LO with sodium borohydride resulted in the series of alcohols **96-102** which all contain an alcohol group at the (n-6) carbon. Soybean 15-LO tolerates the β -ether, β -thioether, γ -thioether, amide and α -branched diacid groups in the synthetic PUFAs **83-89** without changing the regioselectivity of enzyme catalysed oxidation.

Oxidation of the synthetic PUFAs **83-89** by potato 5-LO was investigated using a spectroscopic assay in the previous Chapter. As shown above for the products formed using 15-LO, it should be possible to use electrospray mass spectrometry to confirm the cases in which 5-LO is catalysing oxidation of the synthetic PUFAs **83-89** and also to identify the regioisomers formed in each case.

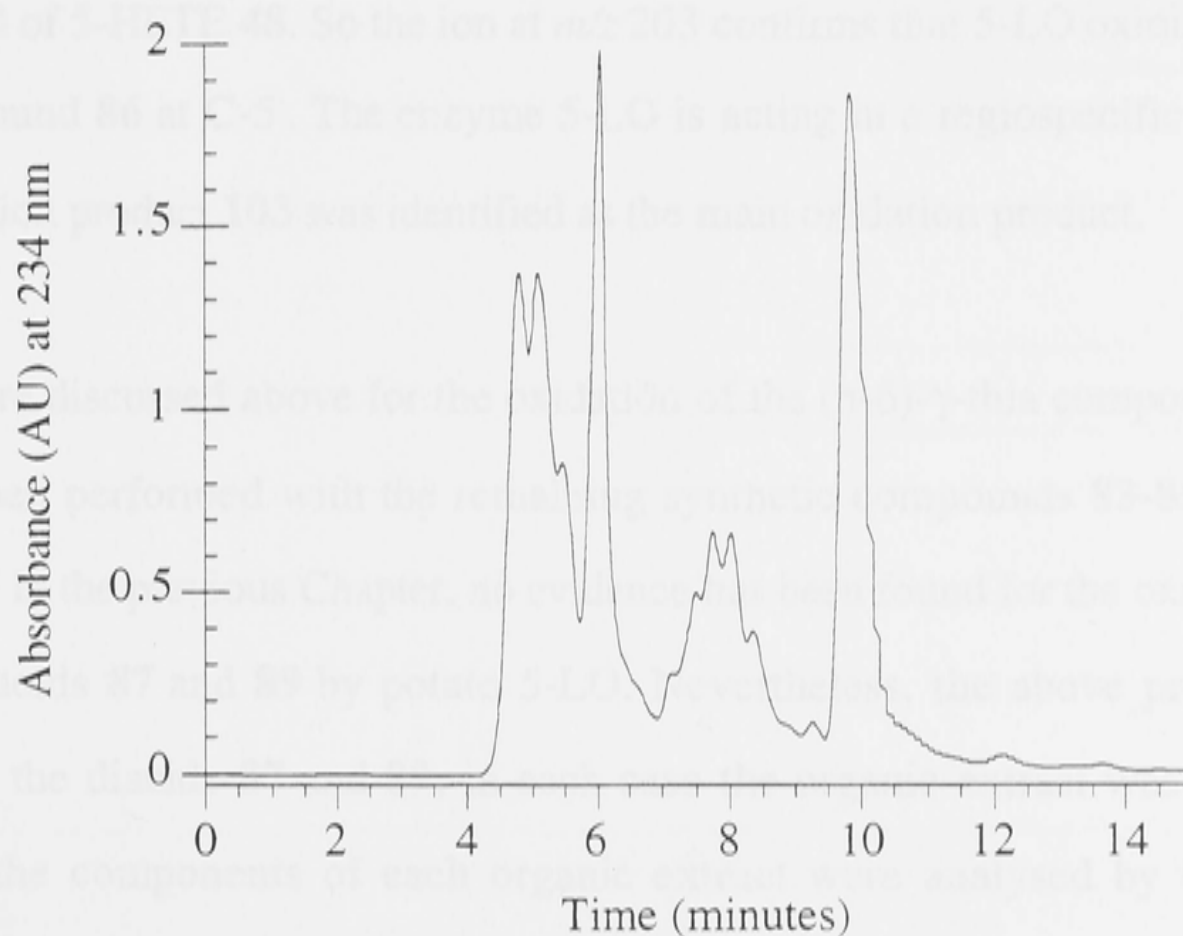
The technique used earlier to isolate oxidation products formed using 15-LO, was adapted on a smaller scale to investigate 5-LO catalysed oxidation of the synthetic PUFAs **83-89**. Potato 5-LO extract (210 μ l) was added to a solution containing the (n-6)- γ -thia compound **86** (100 μ M) in buffer (2.48 ml, pH 5.8, 0.1 M KH_2PO_4 , 0.01% Brij58 detergent) and the mixture was kept at 35 °C for 20 minutes. Sodium borohydride (0.5 mg) was dissolved in the buffer, after 30 minutes the sample was acidified with dilute

Fig 5.9: The electrospray MS/MS spectrum for the product **102** from the oxidation of the (n-6)- α -branched amide **89** by 15-LO



hydrochloric acid and the product was extracted into dichloromethane (2 x 5 ml). The solvent was evaporated, the residue was resuspended in HPLC solvent (200 μ l; hexane : isopropanol : acetic acid, 980 : 20 : 1) and analysed by HPLC as shown in Fig 5.10. Whereas a single peak was detected at 234 nm for the mixture obtained by oxidation of the (n-6)- γ -thia compound **86** with 15-LO, a number of products with retention times between 4.5 and 10 minutes were detected with the mixture obtained following oxidation of the same compound **86** with potato 5-LO. The mixture formed using 5-LO was split into three fractions containing the components with retention times between 4-5.8 minutes (fraction A), 5.8-9.5 minutes (fraction B) and 9.5-10.5 minutes (fraction C). Each fraction was analysed by negative ion electrospray mass spectrometry. Fractions A and B were not analysed further because only fraction C contained material with a molecular ion

Fig 5.10: HPLC of the products derived from oxidation of the (n-6)- γ -thia compound **86** using 5-LO

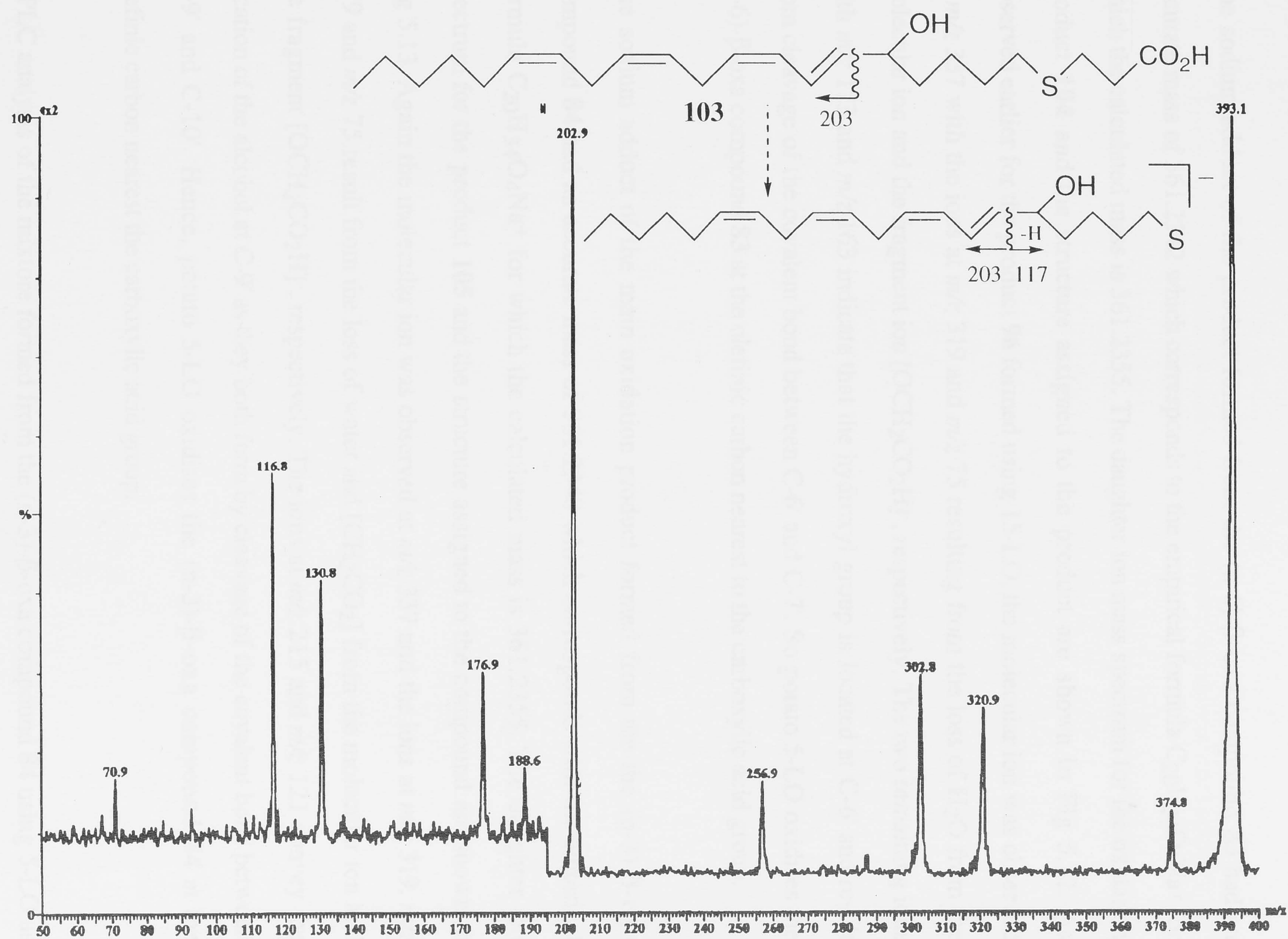


corresponding to an oxidation product derived from the (n-6)- γ -thia compound **86**. The accurate mass was 417.2442 for the sodium adduct of the product in fraction C and corresponds with the empirical formula $C_{23}H_{38}O_5SNa^+$ for which the calculated accurate mass is 417.2439. Hence, the product formed by oxidation of the (n-6)- γ -thia compound **86** using 5-LO has the same empirical formula as the oxidation product formed using 15-LO. Unlike the product **99** formed with 15-LO, the product of 5-LO catalysed oxidation did not decompose during prolonged storage.

The negative ion tandem electrospray mass spectrum and the structure assigned to the compound **103** on the basis of the mass spectrum are shown in Fig 5.11. The molecular anion is at m/z 393 and the daughter ions at m/z 375, m/z 321 and m/z 303 form by the loss of H_2O , $CH_2CH_2CO_2$ and both H_2O and $CH_2CH_2CO_2$, respectively, from the molecular ion. An ion at m/z 203 was observed in the daughter ion spectrum for 5-HETE **48** so the presence of this ion with the formula $C_{15}H_{23}$ in Fig 5.11 indicates that the alcohol is at C-5. The same ion can form from the oxidation product **103** because the ion forms from the lipophilic tail of the molecule where the structure is identical to the lipophilic tail of 5-HETE **48**. So the ion at m/z 203 confirms that 5-LO oxidises the (n-6)- γ -thia compound **86** at C-5'. The enzyme 5-LO is acting in a regiospecific manner as a single oxidation product **103** was identified as the main oxidation product.

The procedure discussed above for the oxidation of the (n-6)- γ -thia compound **86** using 5-LO was then performed with the remaining synthetic compounds **83-85** and **87-89**. As discussed in the previous Chapter, no evidence has been found for the oxidation of the branched diacids **87** and **89** by potato 5-LO. Nevertheless, the above procedure was repeated for the diacids **87** and **89**, in each case the organic extract was analysed by HPLC and the components of each organic extract were analysed by negative ion electrospray mass spectrometry. No molecular ion corresponding to oxidation products from either compound was observed but for an HPLC fraction from each incubation mixture a molecular ion was observed for the unoxidised synthetic PUFA. So although

Fig 5.11: The electrospray MS/MS spectrum for the product **103** from the oxidation of the (n-6)- γ -thia compound **86** by 5-LO



soybean 15-LO oxidises the branched diacids **87** and **89**, neither compound is a substrate for potato 5-LO.

The sodium adduct of the product formed from the (n-6)- β -oxa compound **83** had an accurate mass of 361.2342 which corresponds to the empirical formula $C_{20}H_{34}O_4Na^+$ for which the calculated mass is 361.2355. The daughter ion mass spectrum for the oxidation product **104** and the structure assigned to the product are shown in Fig 5.12. As observed earlier for the product **96** formed using 15-LO, the molecular ion was observed at m/z 337 with the ions at m/z 319 and m/z 75 resulting from the loss of H_2O from the molecular ion and the fragment ion $[OCH_2CO_2H]^-$, respectively. The two remaining ions with m/z 173 and m/z 163 indicate that the hydroxyl group is located at C-6' and result from cleavage of the covalent bond between C-6' and C-7'. So potato 5-LO oxidises the (n-6)- β -oxa compound **83** at the olefinic carbon nearest to the carboxylic acid group.

The sodium adduct of the main oxidation product formed from the (n-3)- β -oxa compound **84** had an accurate mass of 361.2348 which corresponds with the empirical formula $C_{20}H_{34}O_4Na^+$ for which the calculated mass is 361.2355. The daughter ion spectrum for the product **105** and the structure assigned to the compound are shown in Fig 5.13. Again the molecular ion was observed at m/z 337 and the ions at m/z 319, m/z 279 and m/z 75 result from the loss of water and $[CH_2CO_2]$ from the molecular ion and the fragment $[OCH_2CO_2H]^-$, respectively. The ions at m/z 215 and m/z 121 convey the location of the alcohol at C-9' as they both form by cleavage of the covalent bond between C-9' and C-10'. Hence, potato 5-LO oxidises the (n-3)- β -oxa compound **84** at the olefinic carbon nearest the carboxylic acid group.

HPLC analysis of the mixture formed from the (n-3)- β -oxa compound **84** using 5-LO as shown in Fig 5.14 indicates that two other products with similar retention times (12 and 13 minutes, respectively) formed in about 10% and 6% of the yield for the main product. One of these minor products had a retention time identical to that observed for the oxidation product **97** formed using 15-LO shown in Fig 5.15. A molecular ion was

Fig 5.12: The electrospray MS/MS spectrum for the product **104** from the oxidation of the (n-6)- β -oxa compound **83** by 5-LO

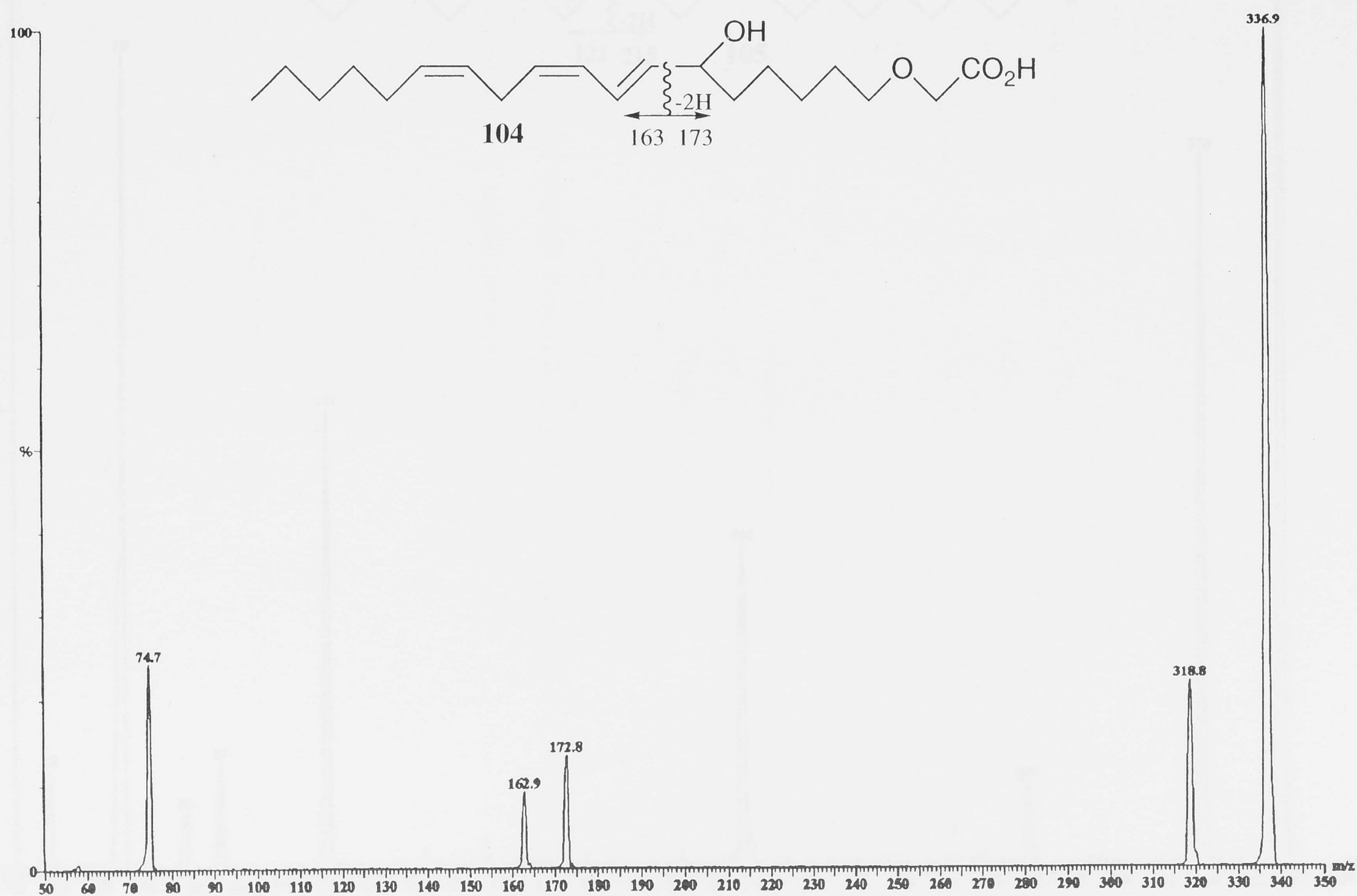


Fig 5.13: The electrospray MS/MS spectrum for the product **105** from the oxidation of the (n-3)- β -oxa compound **84** by 5-LO

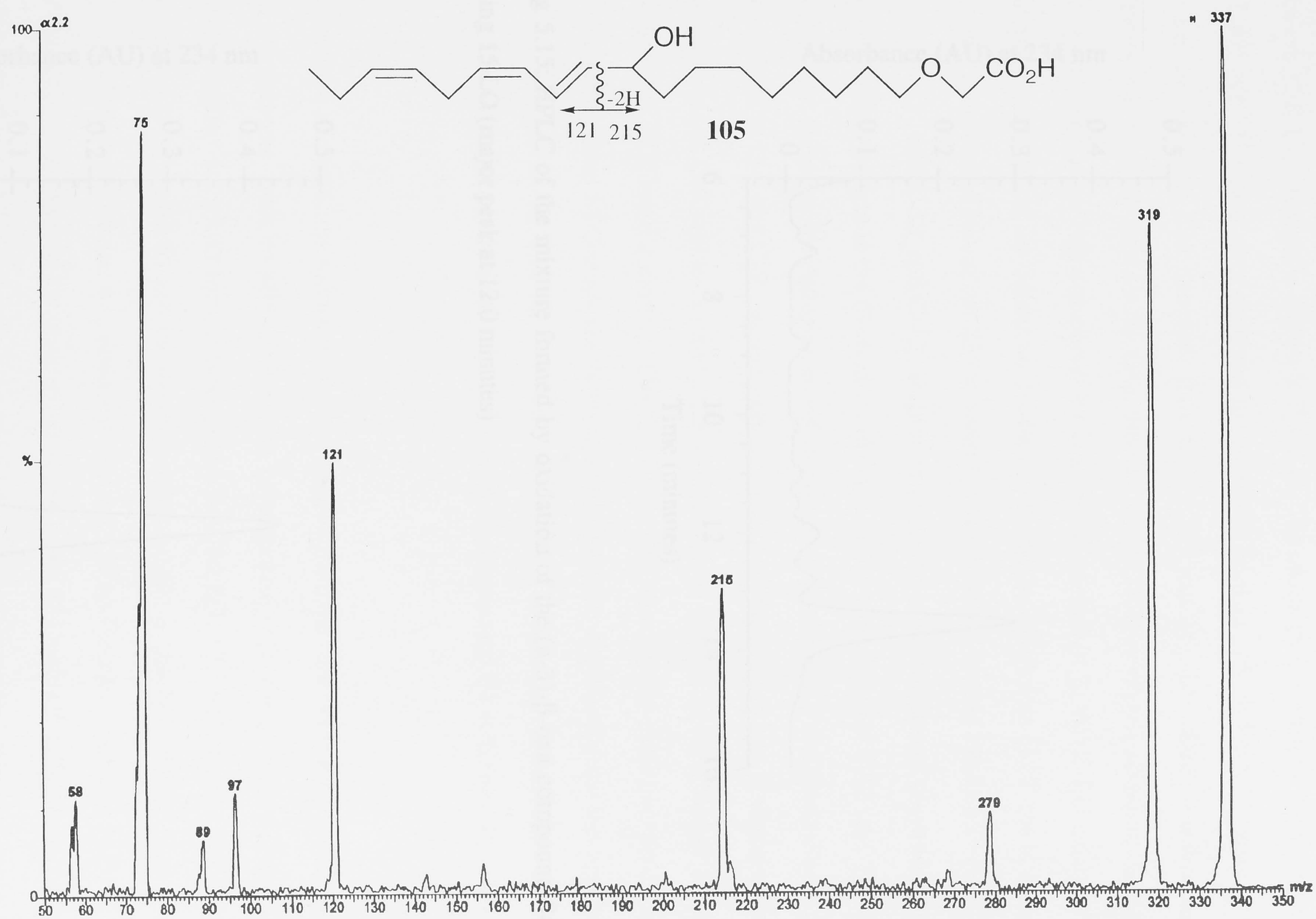


Fig 5.14: HPLC of the mixture formed by oxidation of the (n-3)- β -oxa compound **84** using 5-LO (major peak at 13.6 minutes)

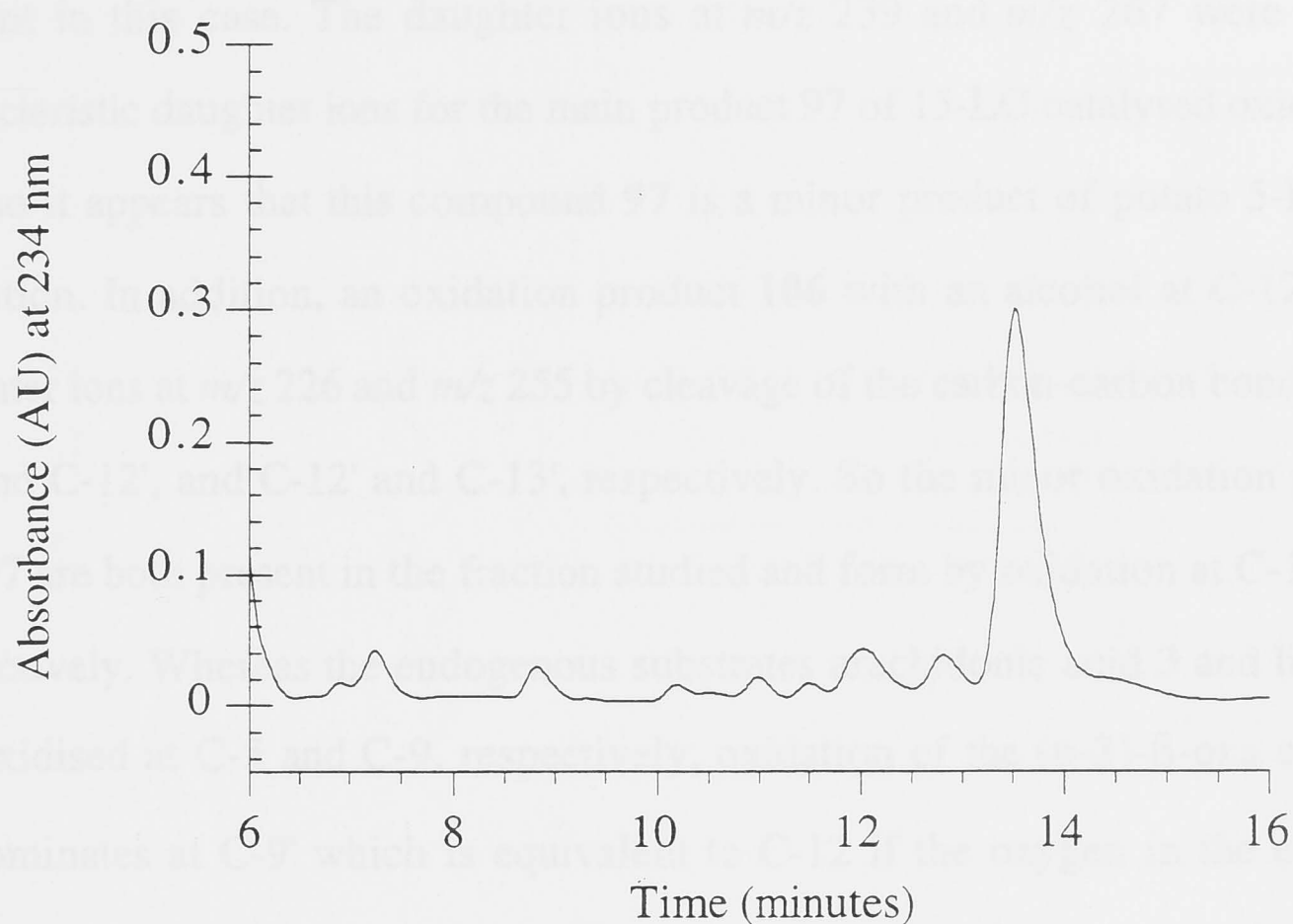
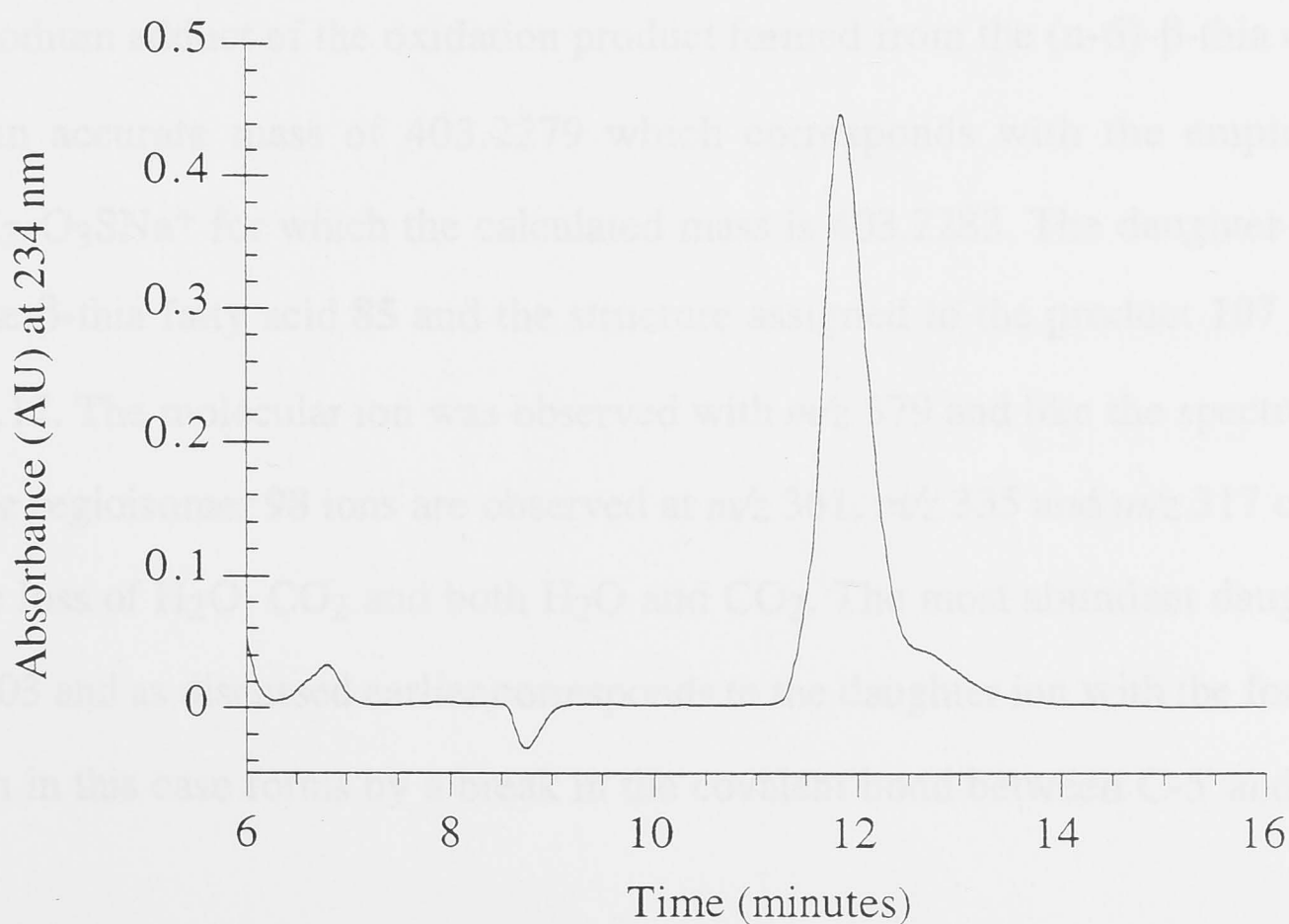


Fig 5.15: HPLC of the mixture formed by oxidation of the (n-3)- β -oxa compound **84** using 15-LO (major peak at 12.0 minutes)



observed at m/z 337 during mass spectrometric analysis of the minor products of 5-LO catalysed oxidation. The daughter ion mass spectrum of the fraction with a retention time between 11.8-12.3 minutes is shown in Fig 5.16. The characteristic daughter ions at m/z 215 and m/z 121 which were observed for the major product **105** (in Fig 5.13) were not present in this case. The daughter ions at m/z 239 and m/z 267 were observed as characteristic daughter ions for the main product **97** of 15-LO catalysed oxidation (in Fig 5.4) so it appears that this compound **97** is a minor product of potato 5-LO catalysed oxidation. In addition, an oxidation product **106** with an alcohol at C-12' could form daughter ions at m/z 226 and m/z 255 by cleavage of the carbon-carbon bonds between C-11' and C-12', and C-12' and C-13', respectively. So the minor oxidation products **106** and **97** are both present in the fraction studied and form by oxidation at C-12' and C-13', respectively. Whereas the endogenous substrates arachidonic acid **3** and linoleic acid **4** are oxidised at C-5 and C-9, respectively, oxidation of the (n-3)- β -oxa compound **84** predominates at C-9' which is equivalent to C-12 if the oxygen in the ether group is numbered as if it were a carbon. The formation of a mixture of oxidation products indicates that potato 5-LO is not absolutely specific in the site of oxidation and this may be due to the poor likeness of the synthetic substrate **84** to the natural substrate **4** and consequently the relatively poor fit of the (n-3)- β -oxa compound **84** at the enzyme active site.

The sodium adduct of the oxidation product formed from the (n-6)- β -thia compound **85** had an accurate mass of 403.2279 which corresponds with the empirical formula $C_{22}H_{36}O_3SNa^+$ for which the calculated mass is 403.2283. The daughter ion spectrum for the β -thia fatty acid **85** and the structure assigned to the product **107** are shown in Fig 5.17. The molecular ion was observed with m/z 379 and like the spectrum in Fig 5.5 for the regioisomer **98** ions are observed at m/z 361, m/z 335 and m/z 317 corresponding to the loss of H_2O , CO_2 and both H_2O and CO_2 . The most abundant daughter ion is at m/z 203 and as discussed earlier corresponds to the daughter ion with the formula $C_{15}H_{23}$ which in this case forms by a break in the covalent bond between C-5' and C-6'. Hence,

Fig 5.16: The electrospray MS/MS spectrum for the minor products **97** and **106** from the oxidation of the (n-3)- β -oxa compound **84** by 5-LO

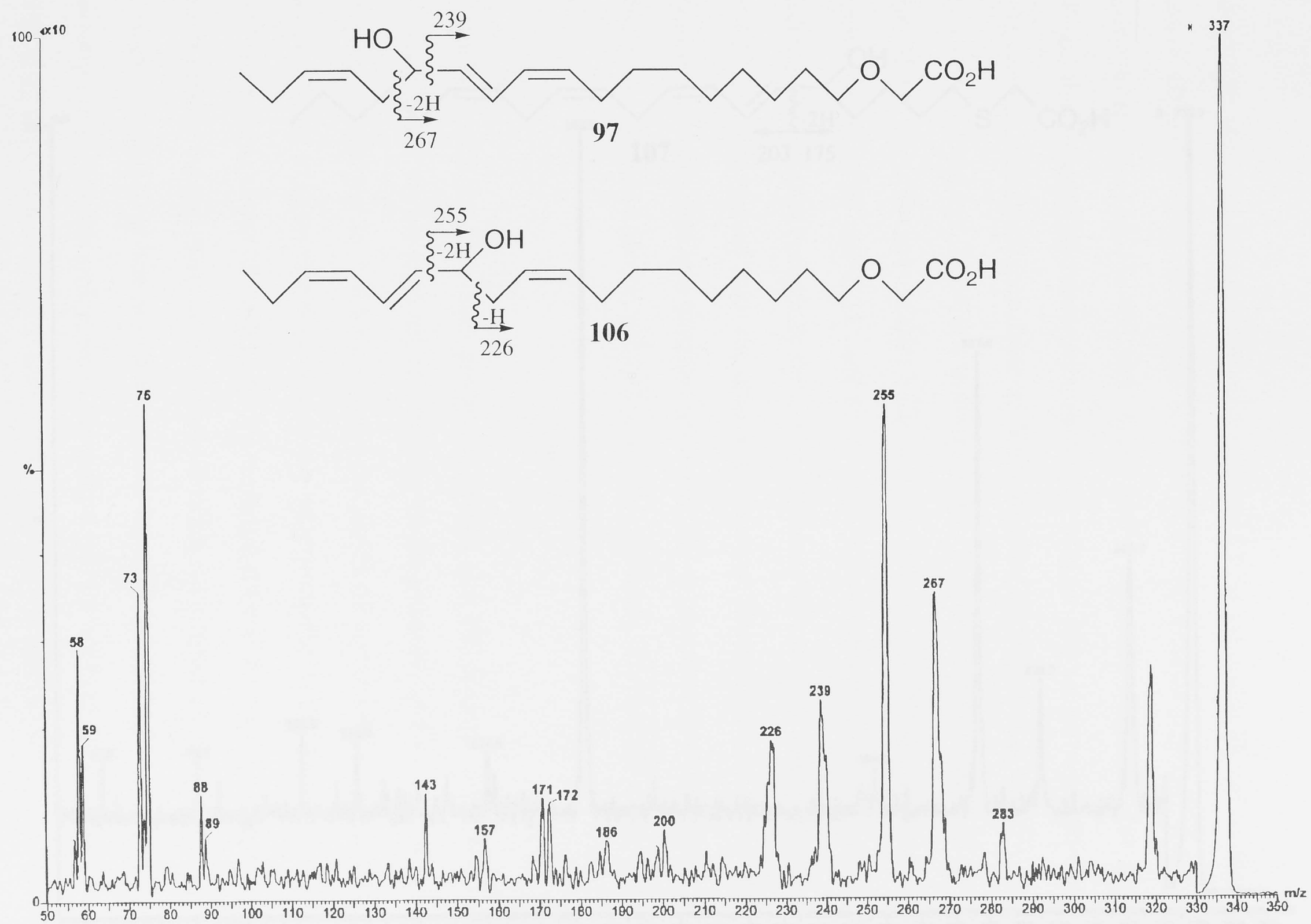
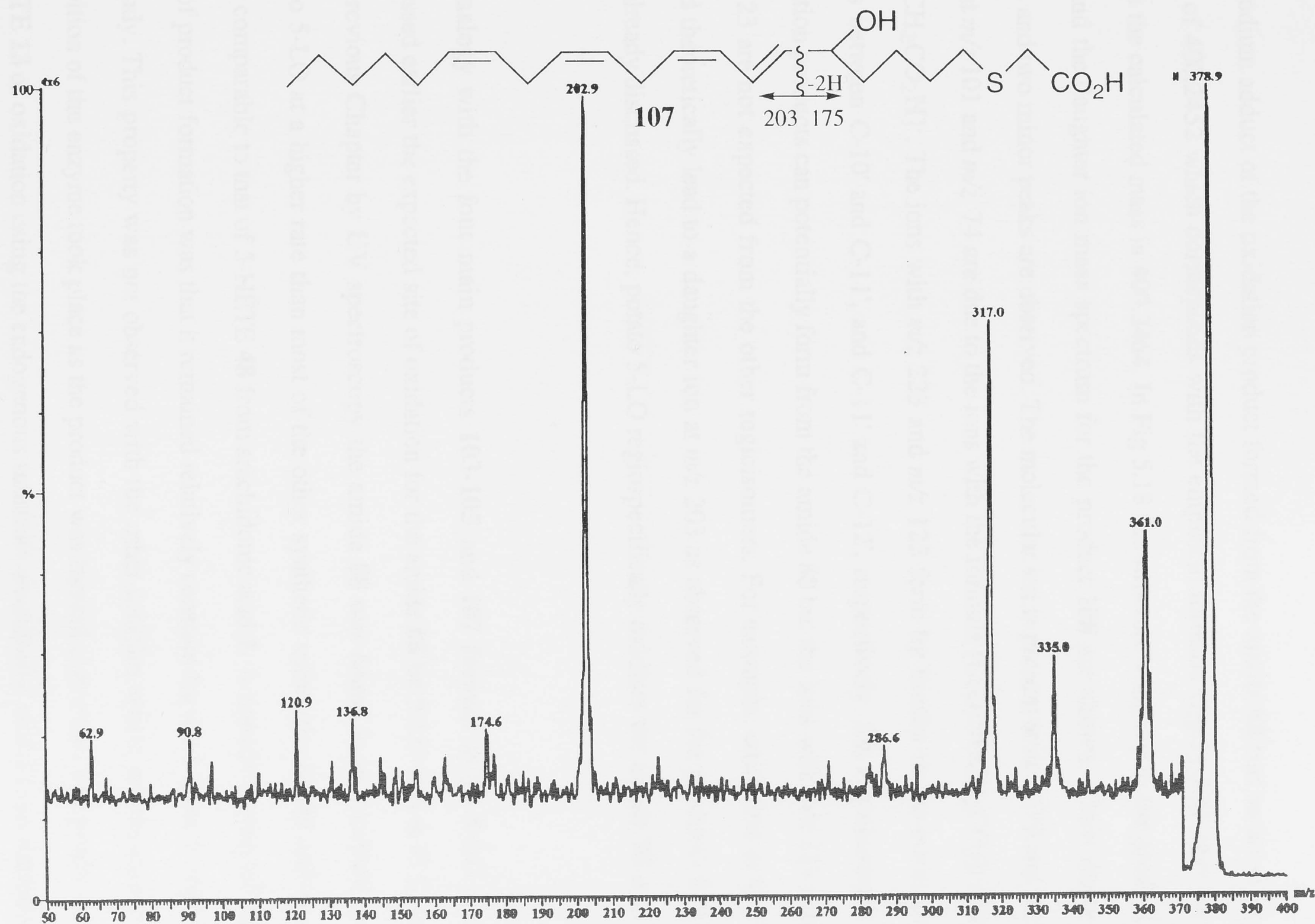


Fig 5.17: The electrospray MS/MS spectrum for the product **107** from the oxidation of the (n-6)- β -thia compound **85** by 5-LO

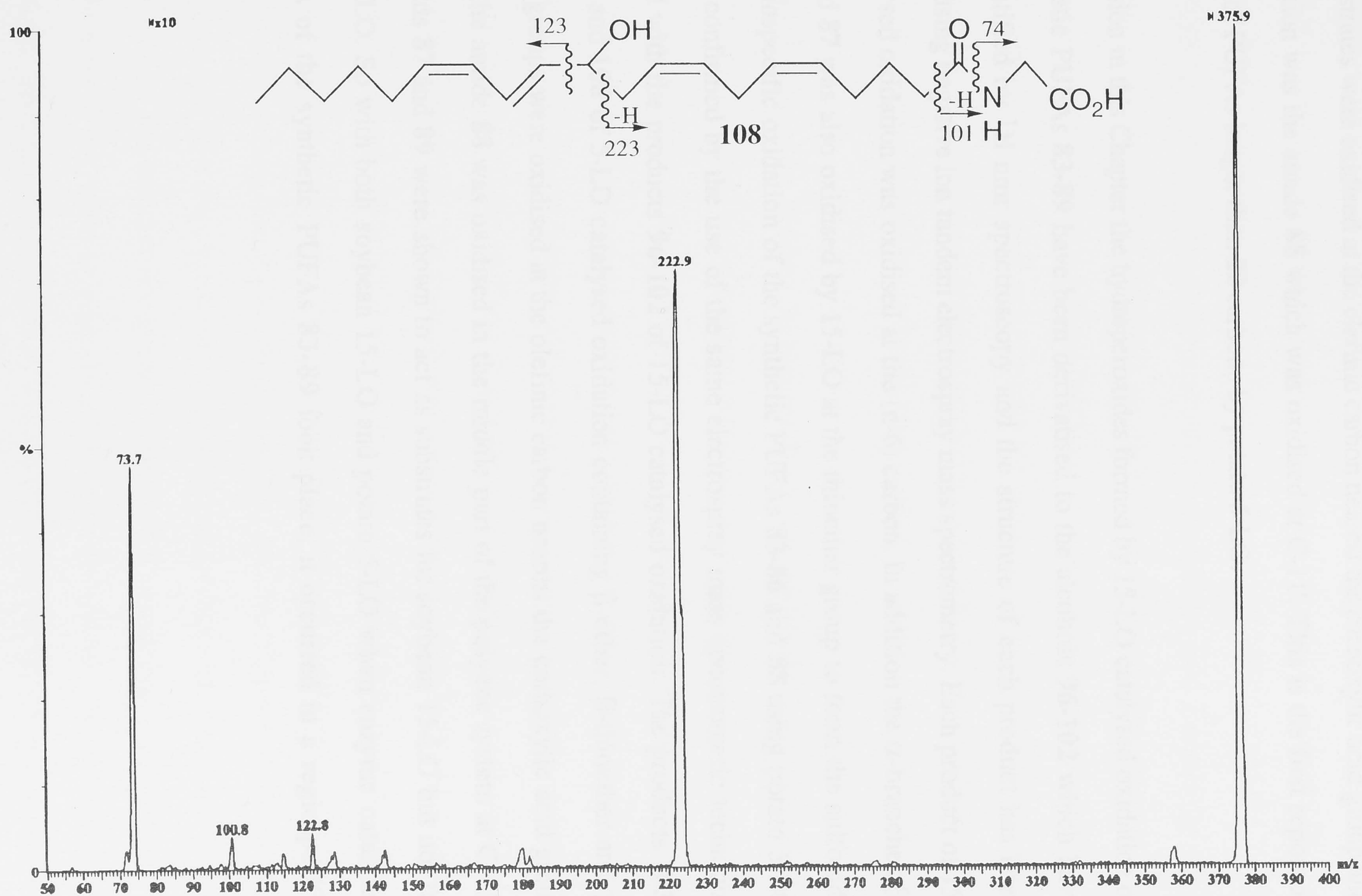


5-LO oxidises the (n-6)- β -thia compound **85** at the olefinic carbon nearest the carboxylic acid group.

The sodium adduct of the oxidation product formed from the amide **88** had an accurate mass of 400.2452 which corresponds with the empirical formula $C_{22}H_{35}NO_4Na^+$ for which the calculated mass is 400.2464. In Fig 5.18 the structure assigned to the product **108** and the daughter ion mass spectrum for the product **108** are shown. Three major peaks and two minor peaks are observed. The molecular ion is present at m/z 379 and the ions at m/z 101 and m/z 74 are due to the ions with the formula $[C(O)NHCH_2CO_2]^-$ and $[NHCH_2CO_2H]^-$. The ions with m/z 223 and m/z 123 form by breaks in the covalent bonds between C-10' and C-11', and C-11' and C-12', respectively. Six regioisomeric oxidation products can potentially form from the amide **88** but the ions with m/z 223 and m/z 123 are not expected from the other regioisomers. For example, oxidation at C-5' would theoretically lead to a daughter ion at m/z 203 as observed for the products **103-106** already discussed. Hence, potato 5-LO regiospecifically oxidises the amide **88** at C-11'.

By analogy with the four main products **103-105** and **107** formed using 5-LO and discussed earlier the expected site of oxidation for the amide **88** by 5-LO was at C-5'. In the previous Chapter by UV spectroscopy the amide **88** was found to be oxidised by potato 5-LO at a higher rate than most of the other synthetic substrates **84-86** and in a yield comparable to that of 5-HETE **48** from arachidonic acid **3**. A notable feature of the rate of product formation was that it remained relatively constant for the 5 minute period of study. This property was not observed with the other samples where inactivation or inhibition of the enzyme took place as the product was formed over time. The product 5-HPETE **13** of oxidation using the endogenous substrate arachidonic acid **3** is an inhibitor of 5-LO. The lack of inhibition caused by the product **108** may result from the unusual site of oxidation at C-11' and consequently a poor resemblance to the endogenous inhibitor 5-HPETE **13**.

Fig 5.18: The electrospray MS/MS spectrum for the product **108** from the oxidation of the (n-6)-amide **88** by 5-LO



Potato 5-LO has been shown to oxidise unbranched synthetic PUFAs containing β -ether, β -thioether, γ -thioether and amide groups in the synthetic PUFAs **83-87** and **88**. Four of the substrates were oxidised at the olefinic carbon nearest the carboxylic acid group and the exception was the amide **88** which was oxidised at C-11'. This is the first report of oxidation of PUFAs longer than 22 carbons by potato 5-LO.

In conclusion in this Chapter the hydroperoxides formed by 15-LO catalysed oxidation of the synthetic PUFAs **83-89** have been derivatised to the alcohols **96-102** which have been identified by ^1H nmr spectroscopy and the structure of each product has been assigned using negative ion tandem electrospray mass spectrometry. Each product of 15-LO catalysed oxidation was oxidised at the (n-6) carbon. In addition the α -branched β -thia diacid **87** was also oxidised by 15-LO at the thioether group to form the sulfoxide **100**. Regiospecific oxidation of the synthetic PUFAs **83-86** and **88** using potato 5-LO has been confirmed by the use of the same electrospray mass spectrometric technique employed with the products **96-102** of 15-LO catalysed oxidation. The products **103-105**, **107** and **108** of 5-LO catalysed oxidation containing β -ether, β -thioether and γ -thioether groups were oxidised at the olefinic carbon nearest the carboxylic acid group whereas the amide **88** was oxidised in the middle part of the polyene system at C-11'. The diacids **87** and **89** were shown to act as substrates for soybean 15-LO but not for potato 5-LO. So with both soybean 15-LO and potato 5-LO when enzyme catalysed oxidation of the synthetic PUFAs **83-89** took place, it occurred in a regiospecific manner.

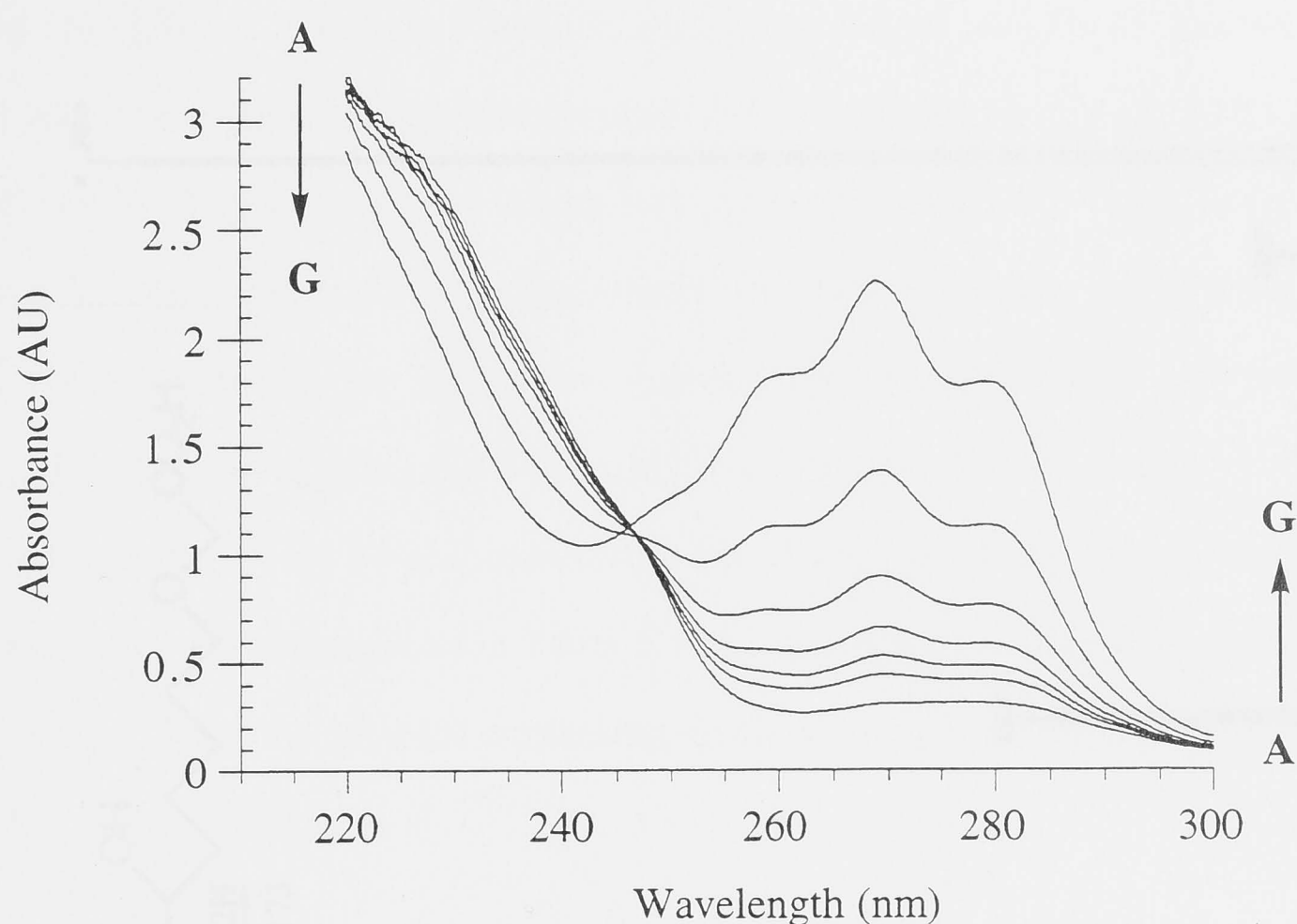
Analysis of the effects of an oxidised β -oxa fatty acid on lipoxygenases

In the previous Chapter it was shown that each of the unbranched synthetic PUFAs **83-86** and **88** is a substrate for both soybean 15-LO and potato 5-LO. The products of arachidonic acid **3** oxidation using 15-LO or 12-LO, (15*S*)-HPETE (*S*)-**14**^{72,73} and (12*S*)-HPETE (*S*)-**46**⁷⁴ have been shown to inhibit arachidonic acid **3** oxidation using 5-LO by acting as competitive substrates for the enzyme. The aim of the work in this section was to determine whether an oxidation product formed from a synthetic compound using 15-LO could act as a substrate for potato 5-LO, to identify any products and to determine whether the oxidation of arachidonic acid **3** by 5-LO is inhibited in the presence of the product formed by oxidation using 15-LO.

As discussed earlier, the product **96** derived from the oxidation of the (n-6)- β -oxa compound **83** using 15-LO and subsequent reduction with sodium borohydride contains an alcohol group bonded to the (n-6) carbon. Using the spectroscopic assay discussed in Chapter 3 to monitor for the presence of the diene functionality at 234 nm, the oxidation product **96** (50 μ M) was incubated with potato 5-LO. A small but steady decrease in absorbance over time was recorded so a drop in the concentration of the conjugated diene **96** was occurring over time. The reaction was repeated and as shown in Fig 6.1 scanning the absorbance between 220 and 300 nm over 64 minutes revealed that the absorbance at 234 nm was falling at -0.016 AU / min whereas the absorbance was increasing between 250-290 nm with maxima forming at 260, 270 and 280 nm. These maxima are consistent with the formation of a conjugated triene group in the product and would result from oxidation at C-6'. The reaction rate is unusual because it is sustained for over an hour whereas in most of the reactions observed earlier with the synthetic compounds **83-86** and **88** enzyme inhibition was apparent by a fall in the rate of product formation after less than 5 minutes.

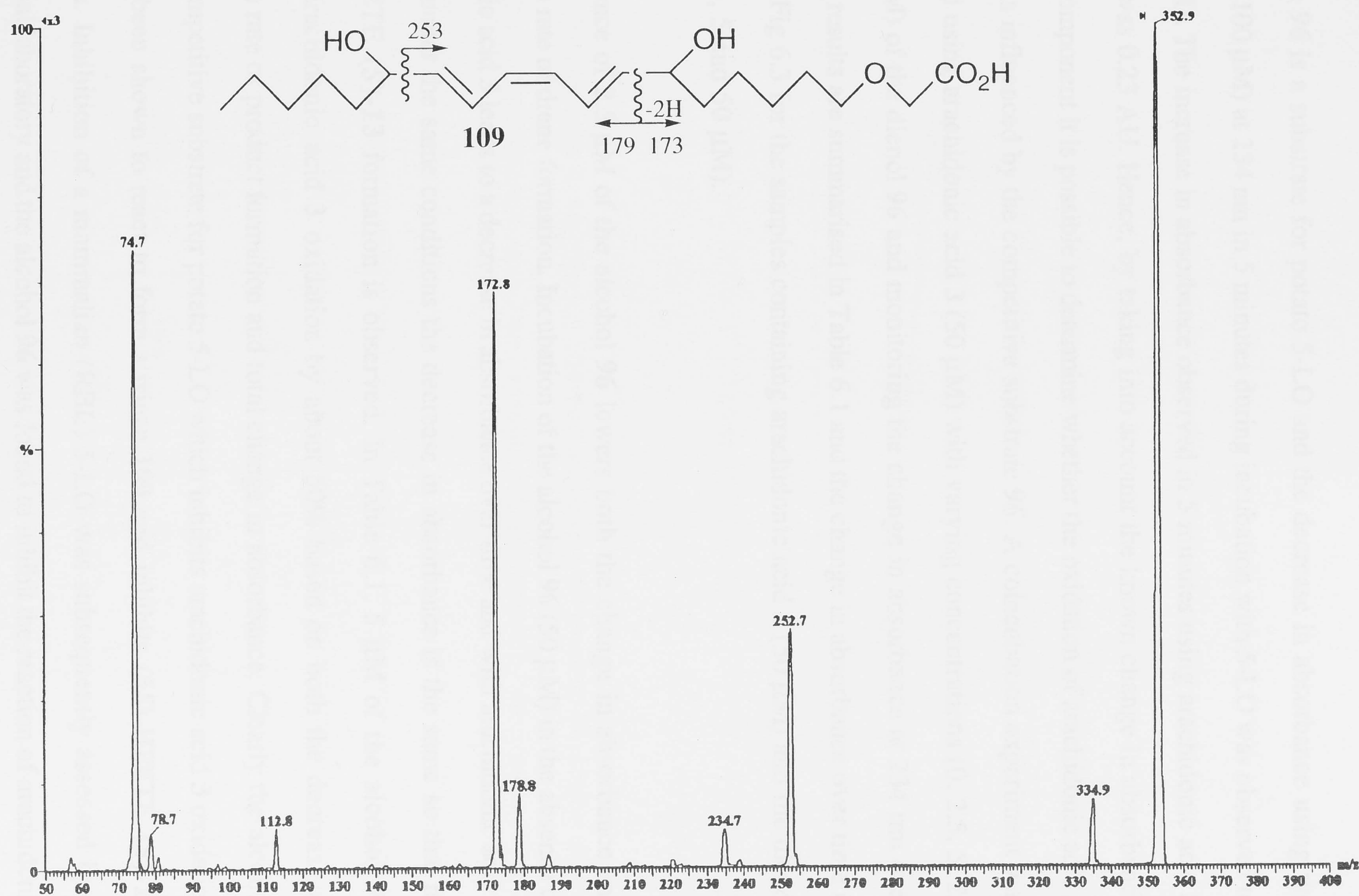
The oxidation product formed above by incubation of the β -oxa-dienol **96** with 5-LO was reduced by incubation with sodium borohydride in the buffer for 45 minutes. Following

Fig 6.1: The absorbance between 220-300 nm after 0.1 (A), 2 (B), 4 (C), 8 (D), 16 (E), 32 (F) and 64 (G) minutes during incubation of the β -oxa-dienol **96** with potato 5-LO for 64 minutes



isolation by HPLC with detection at 270 nm, the triene was analysed using mass spectrometry. By high resolution positive ion mass spectrometry, the accurate mass recorded for the sodium adduct of the product was 377.2303 and corresponded with the empirical formula $C_{20}H_{34}O_5Na^+$ for which the calculated mass is 377.2304. Hence, the product has two oxygens more than the (n-6)- β -oxa compound **83** and one oxygen more than the 15-LO oxidation product **96**. The negative ion tandem electrospray mass spectrum is shown in Fig 6.2 with the structure assigned to the product **109**. The molecular anion is present at m/z 353 and loses H_2O to form the ion at m/z 335. The ion at m/z 75 corresponds with the formula $[OCH_2CO_2H]^-$ and was present in Fig 5.12 as well as the peak at m/z 173 for the oxidation product **96** formed from the (n-6)- β -oxa compound **83** using 5-LO. The ions at m/z 173 and m/z 179 result from a break in the bond between C-6' and C-7' and indicate that oxidation has occurred at C-6'. The ion at m/z 253 results from a break in the bond between C-12' and C-13' and indicates that a second hydroxyl group is present at C-13'. So potato 5-LO oxidised the β -oxa-dienol **96**

Fig 6.2: The electrospray MS/MS spectrum for the product **109** from the oxidation of the β -oxa dienol **96** by 5-LO



at C-6' like the 5-LO catalysed oxidation of the (n-6)- β -oxa compound **83** from which the dienol **96** is derived.

The diene **96** is a substrate for potato 5-LO and the decrease in absorbance using the diene **96** (100 μ M) at 234 nm in 5 minutes during incubation with 5-LO was observed to be 0.10 AU. The increase in absorbance observed in 5 minutes using arachidonic acid **3** (50 μ M) was 0.23 AU. Hence, by taking into account the known change in absorbance for each component it is possible to determine whether the oxidation of arachidonic acid **3** by 5-LO is influenced by the competitive substrate **96**. A coincubation experiment was performed using arachidonic acid **3** (50 μ M) with varying concentrations (0, 2.5, 5, 25 and 50 μ M) of the dienol **96** and monitoring the change in absorbance at 234 nm over time. The results are summarised in Table 6.1 and the change in absorbance over time is shown in Fig 6.3 for the samples containing arachidonic acid **3** (50 μ M) and the dienol **96** (0, 2.5, 5 and 50 μ M).

The presence of 2.5 μ M of the alcohol **96** lowers both the change in absorbance and maximum rate of diene formation. Incubation of the alcohol **96** (50 μ M) in the absence of arachidonic acid **3** leads to a decrease in absorbance over time and with arachidonic acid **3** (50 μ M) under the same conditions the decrease in absorbance is the same so that no (5*S*)-HPETE (*S*)-**13** formation is observed. In Table 6.1, 5 μ M of the alcohol **96** inhibits arachidonic acid **3** oxidation by about 50% based on both the decrease in maximum rate of product formation and total change in absorbance. Clearly the alcohol **96** is a competitive substrate for potato 5-LO which inhibits arachidonic acid **3** oxidation as it has been shown to react to form a triene **109** and inhibits (5*S*)-HPETE (*S*)-**13** formation. Inhibition of a mammalian (RBL) 5-LO was subsequently assessed by a commercial laboratory and the alcohol **96** was found to inhibit the reaction of arachidonic acid **3** with an IC_{50} equal to 3.7 μ M.¹²⁰

Fig 6.3: The change in absorbance over time during the incubation of arachidonic acid **3** (50 μM) with potato 5-LO in the presence of 0 (**A**), 2.5 (**B**), 5 (**C**) and 50 μM (**D**) of the β -oxa-dienol **96**

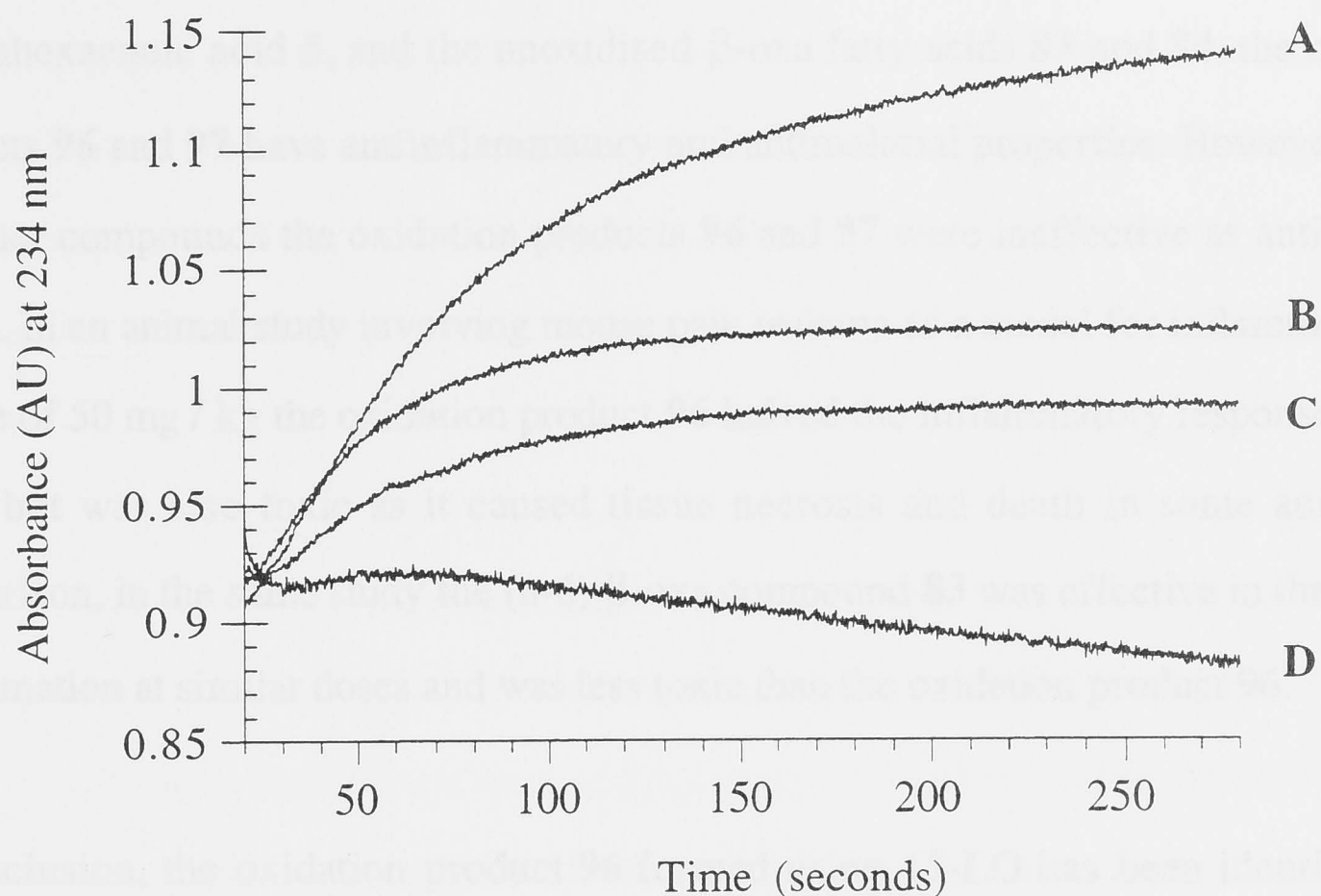


Table 6.1: The change in absorbance and maximum rate of product formation during coincubation of arachidonic acid **3** and various concentrations of the diol **96** with potato 5-LO

[diol 96] μM	ΔAbs	% ΔAbs	V_{max} (nMs^{-1})	% V_{max}
0	0.23	100%*	101	100%*
2.5	0.11	48%	87	87%
5	0.08, 0.04	36%, 21%	53, 43	53%, 43%
25	0.07	29%	46	46%
50	-0.04**	-	-	-

*arbitrarily assigned as 100%

** A virtually identical decrease in absorbance over time was observed during incubation of the diol **96** (50 μM) and arachidonic acid **3** (50 μM) with 5-LO or under the same conditions in the absence of arachidonic acid **3**

At the Adelaide Women's and Children's Hospital and at the Peptech Laboratories further investigations have been made by our colleagues concerning the biological activity of the β -oxa oxidation products **96** and **97** derived from the synthetic β -oxa PUFAs **83** and **84** using 15-LO.¹¹⁸ Like the naturally occurring PUFAs arachidonic acid **3** and docosahexaenoic acid **5**, and the unoxidised β -oxa fatty acids **83** and **84**, the oxidation products **96** and **97** have antiinflammatory and antimalarial properties. However, unlike the other compounds the oxidation products **96** and **97** were ineffective as antiinfective agents. In an animal study involving mouse paw oedema as a model for inflammation at a dosage of 50 mg / kg the oxidation product **96** halved the inflammatory response after 24 hours but was also toxic as it caused tissue necrosis and death in some animals. In comparison, in the same study the (n-6)- β -oxa compound **83** was effective in the relief of inflammation at similar doses and was less toxic than the oxidation product **96**.

In conclusion, the oxidation product **96** formed using 15-LO has been identified as a substrate for potato 5-LO and the triene oxidation product **109** has been identified by mass spectrometry. In a competitive oxidation study using arachidonic acid **3** and the oxidation product **96** with potato 5-LO the competitive substrate **96** was shown to inhibit arachidonic acid **3** oxidation. This result has been confirmed by an independent laboratory with mammalian RBL 5-LO. A study by workers at the Peptech Laboratories has indicated that *in vivo* the oxidation product **96** relieves inflammation in mice but undesirable effects including tissue necrosis and death accompany the relief of inflammation.

Conclusion

The results presented and discussed in this thesis indicate that soybean 15-LO and potato 5-LO regioselectively oxidise synthetic PUFAs containing a range of functional groups. Tandem electrospray mass spectrometry was used to distinguish between the six regioisomeric oxidation products **48-53** formed by autoxidation of arachidonic acid **3** and subsequently was used to distinguish between the pairs of regioisomeric oxidation products formed separately from the synthetic PUFAs **83-86** and **88** with the two LO enzymes.

Coincubation experiments between arachidonic acid **3** and one of the synthetic PUFAs **83**, **84** or **86** using 15-LO or 5-LO confirmed that the synthetic PUFAs **84** and **86** were consumed over time by both enzymes and the compound **83** is a substrate for 15-LO. In each case, the synthetic compound **83**, **84** or **86** altered the rate of arachidonic acid **3** oxidation by acting as a competitive substrate or by allosterically activating the activity of potato 5-LO.

Spectroscopic studies indicated that during incubation of the synthetic compounds **83-89** with either 5-LO or 15-LO products containing a conjugated diene moiety formed in most cases. The maximum rates of conjugated diene formation from the synthetic PUFAs **83-86** and **88** were similar to the maximum rates of conjugated diene formation during the oxidation of arachidonic acid **3** with 15-LO. In contrast, the maximum rates of conjugated diene formation from the synthetic PUFAs **83-86** and **88** with 5-LO were significantly slower than the maximum rates of conjugated diene formation during the oxidation of arachidonic acid **3** with 5-LO. The branched diacids **87** and **89** were not oxidised by potato 5-LO and formed conjugated dienes relatively slowly with 15-LO.

Product analysis demonstrated that each of the synthetic PUFAs **83-89** was oxidised at the (n-6) carbon by 15-LO indicating that the presence of the β -ether, β -thioether, γ -thioether, α -branched β -thioether, amide or α -branched amide moieties did not prevent

binding of the compounds **83-89** to the enzyme active site. During incubation with 15-LO, the α -branched β -thioether **87** was oxidised at both the (n-6) carbon and at the sulfur to form a sulfoxide. Potato 5-LO did not oxidise the branched diacids **87** and **89** but oxidised the unbranched PUFAs **83-86** and **88** containing the β -ether, β -thioether and γ -thioether groups at the olefinic carbon nearest the carboxylic acid group. Based on the daughter ions observed by tandem mass spectrometry, the oxidation product formed from the amide **88** using 5-LO resulted from oxidation at C-11'.

The 2*E*,4*Z*-dienol **96** was formed by 15-LO catalysed oxidation of the β -oxa compound **83** to a hydroperoxide and subsequent reduction of the hydroperoxide with sodium borohydride. This product **96** was oxidised to a conjugated triene during incubation with potato 5-LO and following reduction with sodium borohydride the diol **109** was identified by tandem electrospray mass spectrometry. The dienol **96** was shown to inhibit the oxidation of arachidonic acid **3** by 5-LO by acting as a competitive substrate for the enzyme.

The oxidation products discussed in this thesis are biologically active and could form *in vivo* by LO catalysed oxidation of the synthetic PUFAs **83-89**. The biological activity of the compounds **83-89** may be associated with metabolites formed by LO catalysed oxidation by mechanisms such as the regulation of enzyme activity for the various LO enzymes present in mammalian cells. The isolation of the oxidation products formed from the synthetic PUFA **83-88** following incubation with cultured cells *in vitro* is currently under investigation by our colleagues.

Experimental

General

Ultraviolet spectra were recorded on a temperature controlled Varian Cary 1E spectrophotometer or Shimadzu UV 160 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on Gemini 200 MHz or 300 MHz spectrometers or a Varian VXR 300 (300 MHz) spectrometer in deuteriochloroform with tetramethylsilane as the internal standard. Chemical shifts are quoted as δ in parts per million downfield from the internal standard. Accurate masses were recorded on a Fisons Instruments AutoSpec mass spectrometer. Negative ion electrospray mass spectra were recorded on a Fisons Instruments Quattro II mass spectrometer. Analytical and semipreparative thin layer chromatography were carried out using Merck Kieselgel 60 F₂₅₄ silica on aluminium backing plates. Photolysis experiments were performed in quartz glass tubes within an Oliphant photochemical reactor. HPLC was performed using a Waters HPLC system with ultra-violet (UV) or refractive index (RI) detection as indicated. HPLC was performed using a Waters μ Porasil Radial-PAK[®] silica cartridge (100 mm x 10 mm) or Waters μ Porasil silica column (5 μm silica, analytical scale: 4.6 x 250 mm, semi-preparative scale: 19 x 300 mm) with the eluents hexane, isopropanol and acetic acid in the ratios indicated in the text or Alltech Spherisorb octadecylsilane (ODS) column (4.6 mm x 250 mm, 3 μm) with the eluents acetonitrile and phosphoric acid (30 mM) solution in the ratios indicated in the text.

Arachidonic acid **3** and docosahexaenoic acid **5** were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). The synthetic fatty acids (all-*Z*)-(octadeca-6,9,12-trienyloxy)acetic acid **83**, (all-*Z*)-(octadeca-9,12,15-trienyloxy)acetic acid **84**, (all-*Z*)-(eicosa-5,8,11,14-tetraenylthio)acetic acid **85**, 3-[(all-*Z*)-(eicosa-5,8,11,14-tetraenylthio)]propionic acid **86**, [all-*Z*-(eicosa-5,8,11,14-tetraenylthiooxo)succinic acid **87** were prepared within our research group by Dr Michael Pitt.¹⁰⁸⁻¹⁰⁹ The amides, *N*-(all-*Z*)-(eicosa-5,8,11,14-tetraenyl)glycine **88** and *N*-(all-*Z*)-(eicosa-5,8,11,14-

tetraenyl)aspartic acid **89** were generously donated by Peptech Ltd, Sydney. Soybean 15-lipoxygenase (150×10^{-6} units / 1.36 g) and Brij[®] 58 (polyoxyethylene (20) cetyl ether) were purchased from Sigma[®] Chemical Company. Ascorbic acid, ammonium sulfate, sodium metabisulfite and sodium hydroxide were purchased from BDH Laboratory Supplies. Hydrochloric acid, potassium dihydrogen ortho-phosphate and salicylic acid were purchased from Ajax Chemicals. Acilit[®], Neutralit[®] and Alkalit[®] pH papers, ethylenediaminetetraacetic acid (EDTA) disodium salt and sodium borohydride were purchased from Merck Chemicals. Triphenylphosphine and Tween[®] 20 (polyoxyethylene (20) sorbitan monolaurate) were purchased from Aldrich[®] Chemical Company.

Buffer solutions

Preparation of pH 9.0 phosphate buffer^{35,52}

Potassium dihydrogen phosphate (13.61 g, 0.1 mol) was dissolved in water (800 ml), the pH was adjusted to 9.0 with the use of sodium hydroxide pellets and the volume was made up to 1000 ml. This buffer was used in the assay of PUFA oxidation using 15-LO.

Preparation of phosphate buffer for the extraction of 5-LO from potatoes^{60,98}

Ascorbic acid (704 mg, 4.0 mmol), sodium metabisulfite (760 mg, 4.0 mmol), EDTA (598 mg, 2.0 mmol) and potassium dihydrogen phosphate (27.21g, 200 mmol) were dissolved in water (1800 ml), the pH was measured and adjusted to 6.0 with sodium hydroxide pellets and the volume was made up to 2000 ml. Grated potatoes were added to an equal volume of this buffer prior to mixing in a Waring blender during the potato 5-LO isolation procedure.

Preparation of phosphate buffer for dialysis of 5-LO extract^{60,98}

Potassium dihydrogen phosphate (10.88 g, 80 mmol) was dissolved in water (1800 ml), the pH was measured and adjusted to 6.3 with sodium hydroxide pellets and the volume was made up to 2000 ml. This buffer was used in the overnight dialysis in crude potato 5-LO extract.

Preparation of phosphate buffer for incubation of 5-LO with PUFAs^{60,98}

Potassium dihydrogen phosphate (0.44 g, 40 mmol) was dissolved in water (800 ml), the pH was measured and adjusted to 6.3 with sodium hydroxide pellets and the volume was made up to 2000 ml. This buffer was used for the incubation of PUFAs with 5-LO in Chapter 2 and with Tween[®] 20 (0.005%) for the spectroscopic study of PUFA oxidation using 5-LO in Chapter 3.

Experimental for Results and Discussion: Chapter 1**(Z,Z,Z,E)-15S-hydroperoxyeicosa-5,8,11,13-tetraenoic acid (S)-14**

Soybean 15-lipoxygenase (7 mg) in buffer (1 ml, pH 9.0, 0.1 M KH_2PO_4) was added to a vigorously stirred solution of arachidonic acid **3** (35 mg, 115 μmol) in the same buffer (50 ml, 25-30 °C). The mixture was stirred for 40 minutes in a 250 ml conical flask whilst oxygen was bubbled through the solution and a stream of nitrogen was used to prevent the build up of froth in the neck of the flask. The product was extracted into cold dichloromethane (2 x 100 ml, 0 °C) following acidification of the buffer using hydrochloric acid (0.2 M, 20 ml). The crude product (S)-**14** (25 mg) was isolated by drying the dichloromethane with magnesium sulphate followed by filtration and evaporation of the organic layer. The solution was kept cold (0 °C) throughout the

extraction and isolation procedure. The product (**S**)-**14** was purified by cold (0 °C) preparative thin layer chromatography (hexane : ether : acetic acid, 40 : 60 : 1) and the silica containing the product (**S**)-**14** was scraped from the TLC plate. (Z,Z,Z,E)-15S-hydroperoxyeicosa-5,8,11,13-tetraenoic acid (**S**)-**14** (21 mg, 57%) was extracted from the silica using ether : methanol (9 : 1) and isolated following evaporation of the solvent.

¹H nmr (300 MHz, CDCl₃) δ 0.88 (3H, m, C-20-H₃), 1.20-1.45 (8H, m), 1.60-1.75 (2H, m), 2.05-2.20 (2H, m), 2.28-2.45 (2H, m), 2.73-2.88 (2H, m), 2.91-3.05 (2H, m), 4.40 (1H, dt, 6.0, 7.0 Hz, C15-H), 5.25-5.48 (5H, m), 5.60 (1H, dd, 7.0, 15.3 Hz, C14'-H), 6.03 (1H, t, 11.1 Hz, C12'-H), 6.60 (1H, dd, 11.0, 15.3 Hz, C13'-H)

Preparation of the HPETE isomers 48-53 by autoxidation of arachidonic acid 3

Arachidonic acid **3** (64 mg, 210 μmol) was mixed with α-tocopherol **15** (5 mg, 12 μmol) in dichloromethane (5 ml) in a round bottomed flask and the solvent was evaporated to leave a residue as a thin film. The flask was flushed with oxygen, sealed and stored in darkness at room temperature for 94 hours. The residue was redissolved in deuteriochloroform (5 ml) and analysed by ¹H nmr spectroscopy. The ratio of the area of the peak at 4.3 ppm to the area of the peak at 0.88 ppm was 7 : 100. The peak at 4.3 ppm corresponds to a hydrogen sharing a carbon with a hydroperoxide group and the peak at 0.88 ppm corresponds to the terminal methyl group of both oxidised and unoxidised hydrocarbons. Based on the peak ratio above, the yield of hydroperoxide is 20 %.

Stability study of (15*S*)-hydroperoxyeicosatetraenoic acid (*S*)-**14** during repeated HPLC

(15*S*)-HPETE (*S*)-**14** (0.25 mg) was injected onto the HPLC system and eluted with a retention time between 40 and 54 minutes using unmodified silica (Waters μ Porasil Radial-PAK®; flow rate 2 ml / min; mobile phase: hexane 100% to hexane : isopropanol : acetic acid 987 : 13 : 1, 30 minute gradient; detection at 234nm). All HPLC fractions (5-60 minutes) were combined, concentrated and reinjected. Five times in total, (15*S*)-HPETE (*S*)-**14** was injected once on the first day, twice on the second day and once on the third and fourth days. The peak for (15*S*)-HPETE (*S*)-**14** was initially well off-scale (1.0 AU) by detection at 234 nm but after four days at room temperature and repeated chromatography only a small broad signal was observed with a maximum signal only 20% of the full scale deflection signal.

Attempted isomerisation of 15(*S*)-HPETE (*S*)-**14**

A stock solution was prepared by dissolving crude (15*S*)-HPETE (*S*)-**14** (15 mg) in hexane : dichloromethane 1 : 1 (2 ml). Aliquots (100 μ l, 700 μ g) of the stock solution were diluted to a volume of 2 ml using hexane, sealed and stored under nitrogen at 40 °C for 0, 1 or 2 days and then reduced by dissolving sodium borohydride (1 mg) in the solution for 45 minutes. By HPLC analysis for the HETE isomers **48-53**, one peak was observed in each of the three samples. This peak had the same retention time as the peak observed using an authentic sample of 15-HETE **53** prepared by reduction of 15(*S*)-HPETE (*S*)-**14** (1 mg) with sodium borohydride (1 mg) in ethanol (5 ml). Hence, no evidence of isomerisation was recorded.

Assay for the formation of 11-HETE **51, 12-HETE **52** and 15-HETE **53** from arachidonic acid **3** with α -tocopherol **15** during autoxidation**

Stock solutions of arachidonic acid **3** (100 mg / ml), α -tocopherol **15** (10 mg / ml) and the external standard salicylic acid (1 mg / ml) were prepared in dichloromethane. In six 50 ml round bottom flasks, dichloromethane (5 ml) and a range of concentrations of α -tocopherol **15** (250, 50, 5, 0.5, 0 μ g) were added to arachidonic acid **3** (5 mg, 16 μ mol, 50 μ l). The solvent was evaporated using a rotary evaporator to leave a thin film which was stored in darkness under an atmosphere of oxygen for three days. Triphenylphosphine (5 mg, 19 μ mol), salicylic acid (0.5 mg, 3.62 μ mol) and dichloromethane (5 ml) were added to each sample and after dissolving the residue, the solvent was evaporated. Hexane (1 ml) was added, the residue was dissolved and an aliquot (100 μ l) was injected onto the HPLC. Normal phase HPLC analysis involved a flow rate of 1 ml / min and UV detection (hexane : isopropanol : acetic acid, 1000 : 7 : 1; Waters Radial-Pak silica). The above experiment was repeated using BHT **93**, ascorbic acid **94** or glutathione **95** in place of α -tocopherol **15**.

The extinction coefficient of salicylic acid was calculated by recording the absorbance at 234 nm for salicylic acid solutions in the above mobile phase. The absorbance values were 0.386, 0.754, 1.143, 1.491 and 1.842 AU at the concentrations 0.05, 0.10, 0.15, 0.20 and 0.25 mM, respectively. From these values, by linear regression the extinction coefficient of salicylic acid was calculated to be $7454 \pm 44 \text{ M}^{-1}$. As the extinction coefficient of each HETE isomer **48-53** is reported to be 25000 M^{-1} , from the HPLC analysis the yield of each HETE isomer can be calculated in each sample relative to the peak for salicylic acid (3.62 μ mol / sample). The results are summarised in Tables 2.1-2.4.

Preparation of the HETE isomers 48-53 by photolysis of arachidonic acid **3** with methylene blue

Arachidonic acid **3** (24 mg, 79 μmol) and methylene blue (9 mg, 28 μmol) were dissolved in methanol (5 ml) in a quartz glass tube. Oxygen was bubbled through the solution for 30 seconds, the tube was sealed under an atmosphere of oxygen and placed in an Oliphant photochemical reactor for 16 hours. The solvent was evaporated from the flask and the residue was redissolved in deuteriochloroform (5 ml) and analysed by ^1H nmr spectroscopy. The ratio of the area of the peak at 4.3 ppm to the area of the peak at 0.88 ppm was 13 : 100. The peak at 4.3 ppm corresponds to a hydrogen sharing a carbon with a hydroperoxide group and the peak at 0.88 ppm corresponds to the terminal methyl group of both oxidised and unoxidised hydrocarbons. Based on the peak ratio above, the yield of hydroperoxide is 40 %.

(*Z,Z,Z,E*)-15*S*-hydroxyeicosa-5,8,11,13-tetraenoic acid (*S*)-**53**^{33,36}

Soybean 15-lipoxygenase (6 mg) in buffer (1 ml, pH 9.0, 0.1 M KH_2PO_4) was added to a vigorously stirred solution of arachidonic acid **3** (34 mg, 112 μmol) in the same buffer (50 ml, 25-30 °C). Oxygen was bubbled through the solution, a stream of nitrogen was used to prevent the build up of froth in the neck of the flask and the mixture was stirred for 40 minutes. Sodium borohydride (10 mg, 264 μmol) was dissolved in the buffer and the solution was stirred for 30 minutes. The product (*S*)-**53** was extracted into dichloromethane (2 x 100 ml) with acidification of the buffer using hydrochloric acid (0.2 M, 20 ml). The crude product (*S*)-**53** (25 mg) was isolated by drying with magnesium sulphate, filtration and evaporation of the organic layer. (*Z,Z,Z,E*)-15*S*-hydroxyeicosa-5,8,11,13-tetraenoic acid (*S*)-**53** was isolated by flash chromatography (ether: hexane: acetic acid 50 : 50 : 1) in 59% yield (21 mg, 66 μmol).

^1H nmr (300 MHz, CDCl_3) δ 0.82 (3H, m, C-20- H_3), 1.20-1.45 (8H, m), 1.60-1.80 (2H, m), 2.05-2.20 (2H, m), 2.30-2.45 (2H, m), 2.73-2.88 (2H, m), 2.91-3.05 (2H, m), 4.19 (1H, dt, 6.0, 6.0 Hz, C15-H), 5.25-5.48 (5H, m), 5.71 (1H, dd, 6.0, 15.0 Hz, C14'-H), 6.00 (1H, dd, 11.0, 11.0 Hz, C12'-H), 6.57 (1H, dd, 11.0, 15.0 Hz, C13'-H)

Negative ion electrospray MS/MS: 319, 301, 257, 219, 175, 121 as shown in Fig 2.15

Positive ion high resolution electrospray MS/MS: $\text{C}_{20}\text{H}_{32}\text{O}_3\text{Na}$, $[\text{M}+\text{Na}]^+$ calculated 343.2249, observed 343.2258; $\text{C}_{20}\text{H}_{31}\text{O}_2$, $[\text{M}-\text{OH}]^+$ calculated 303.2324, observed 303.2330.

Isolation of the HETE isomers 49-53 by autoxidation of arachidonic acid **3** with α -tocopherol 15^{29,31}

Arachidonic acid **3** (42 mg, 138 μmol) and α -tocopherol **22** (5 mg, 12% by weight) in dichloromethane were evaporated in a 250 ml round bottom flask. The residue was stored under oxygen in darkness at room temperature for 42 hours and then dissolved in ethanol (5 ml) with sodium borohydride (5 mg, 132 μmol) for an hour. Following evaporation of the solvent and resuspension of a small portion of the residue in mobile phase (hexane : isopropanol : acetic acid, 986 : 13 : 1), the isomers were resolved by analytical HPLC over a period of 50 minutes (flow rate 0.6 ml / min, Alltech Porasil silica, detection at 234 nm). Subsequently, the autoxidation residue (54 mg) was redissolved in isopropanol (200 μl) and HPLC mobile phase (1.8 ml; hexane : isopropanol : acetic acid, 919 : 80 : 1) and separated on a semipreparative scale. The HETE isomers **49-53** were isolated in approximately 1% yield of each isomer (flow rate 9 ml / min). The yield was reduced by blockage of the HPLC system resulting from the poor solubility of some autoxidation products. The retention times observed for the regioisomers **49-53** were: 12-HETE **52** 12.5 minutes, 15-HETE **53** 13.6 minutes, 11-HETE **51** 18.9 minutes, 9-HETE **50** 26.8 minutes and 8-HETE **49** 32.5 minutes as shown in Figs 2.2-2.6.

By ^1H nmr spectroscopy additional signals were observed between 0.5-4.0 ppm which made the complete assignment of all signals impossible without additional purification. Less than 2 mg of each regioisomer **49-53** was isolated and as the olefinic signals observed in the ^1H nmr spectra (and shown in Table 2.5) corresponded with the published olefinic signals for the regioisomers 8-MeHETE, 9-MeHETE, 11-MeHETE and 12-MeHETE,⁴² the samples containing the compounds **49-53** were then characterised by negative ion tandem electrospray mass spectrometry.

(*Z,E,Z,Z*)-8-hydroxyeicosa-5,9,11,14-tetraenoic acid **49**: Negative ion electrospray MS/MS: 319, 301, 257, 163, 155, 127 as shown in Fig 2.11. Positive ion high resolution electrospray MS/MS: $\text{C}_{20}\text{H}_{32}\text{O}_3\text{Na}$, $[\text{M}+\text{Na}]^+$ calculated 343.2249, observed 343.2248; $\text{C}_{20}\text{H}_{31}\text{O}_2$, $[\text{M}-\text{OH}]^+$ calculated 303.2324, observed 303.2330.

(*Z,E,Z,Z*)-9-hydroxyeicosa-5,7,11,14-tetraenoic acid **50**: Negative ion electrospray MS/MS: 319, 301, 179, 167, 151, 123, 69, 59 as shown in Fig 2.12. Positive ion high resolution electrospray MS/MS: $\text{C}_{20}\text{H}_{32}\text{O}_3\text{Na}$, $[\text{M}+\text{Na}]^+$ calculated 343.2249, observed 343.2244.

(*Z,Z,E,Z*)-11-hydroxyeicosa-5,8,12,14-tetraenoic acid **51**: Negative ion electrospray MS/MS: 319, 301, 167, 149, 59 as shown in Fig 2.13. Positive ion high resolution electrospray MS/MS: $\text{C}_{20}\text{H}_{32}\text{O}_3\text{Na}$, $[\text{M}+\text{Na}]^+$ calculated 343.2249, observed 343.2244.

(*Z,Z,E,Z*)-12-hydroxyeicosa-5,8,10,14-tetraenoic acid **52**: Negative ion electrospray MS/MS: 319, 301, 257, 208, 179, 59 as shown in Fig 2.14. Positive ion high resolution electrospray MS/MS: $\text{C}_{20}\text{H}_{32}\text{O}_3\text{Na}$, $[\text{M}+\text{Na}]^+$ calculated 343.2249, observed 343.2238.

(*Z,Z,Z,E*)-15-hydroxyeicosa-5,8,11,13-tetraenoic acid **53**: Negative ion electrospray MS/MS: 319, 301, 257, 219, 175, 59 as shown in Fig 2.14. Positive ion high resolution electrospray MS/MS: $\text{C}_{20}\text{H}_{32}\text{O}_3\text{Na}$, $[\text{M}+\text{Na}]^+$ calculated 343.2249, observed 343.2258; $\text{C}_{20}\text{H}_{31}\text{O}_2$, $[\text{M}-\text{OH}]^+$ calculated 303.2324, observed 303.2330.

Preparation of Potato 5-LO

Potatoes were obtained from commercial sources. At 4 °C whole potatoes were first grated then added to a Waring blender with an equal volume of buffer A (pH 5.5, 100 mM KH₂PO₄, 2 mM Na₂S₂O₇, 1 mM EDTA, 2 mM ascorbic acid **94**, 3 mM Brij58) and blended for 1 minute. The crude extract was filtered through cheese cloth and the clear solution was brought to 30% saturation with ammonium sulphate (21.1 g / 100 ml) and stirred for an hour. The solution was centrifuged (9000 rpm, 30 min) and the liquid was brought to 50% saturation with ammonium sulphate (35.2 g / 100 ml) and stirred for an hour. The cloudy solution was centrifuged (9000 rpm, 30 min) and the solid residue was separated and dissolved in a second buffer (20 ml, pH 6.0, 40 mM KH₂PO₄). This solution was dialysed overnight against the second buffer (2000 ml) and stored at 4 °C until required. For spectrophotometric measurements the crude enzyme extract was centrifuged (9000 rpm, 15 min) prior to use, in order to lower the background absorbance at 234 nm.

(*E,Z,Z,Z*)-5*S*-hydroxyeicosa-6,8,11,14-tetraenoic acid (*S*)-**48**

Arachidonic acid **3** (50 µM, 38 µg) was dissolved in buffer (2.45 ml, pH 9.0, 0.1 M KH₂PO₄) in a quartz cuvette at 30 °C. Crude potato 5-LO (50 µl) was added and the formation of the product (*S*)-**13** was confirmed by monitoring the formation of conjugated diene as the increase in absorbance over time at 234 nm. The major product (*S*)-**13** was reduced to the alcohol (*S*)-**48** by dissolving sodium borohydride (1 mg) in the buffer for 30 minutes, acidified with dilute hydrochloric acid and extracted into dichloromethane (2 x 5 ml). The organic extract was evaporated under nitrogen, resuspended in HPLC mobile phase (100 µl, hexane : isopropanol : acetic acid, 950 : 50 : 1) and analysed by analytical scale HPLC (flow rate 1.5 ml / min, Alltech µPorasil) with detection at 234 nm. The retention time was 13 minutes for (5*S*)-HETE (*S*)-**48**.

compared with 7 minutes for (15*S*)-HETE (**S**)-**53** under the same conditions as shown in Figs 2.8 and 2.9.

Negative ion electrospray MS/MS: 319, 301, 257, 203, 177, 141, 115, 59 as shown in Fig 2.10. Positive ion high resolution electrospray MS/MS: $C_{20}H_{32}O_3Na$, $[M+Na]^+$ calculated 343.2249, observed 343.2238.

Experimental for Results and Discussion: Chapter 2

Competitive autoxidation assay: the 21:3(n-6) β -oxa compound **83** and arachidonic acid **3**

A stock solution was prepared by dissolving arachidonic acid **3** (10 mg), the 21:3(n-6) β -oxa compound **83** (8 mg) and lauric acid (6 mg) in dichloromethane (1 ml). Stock solution (100 μ l) was added to three round bottomed flasks. Dichloromethane (5 ml) was added to each flask, α -tocopherol **15** (0.1 mg) and azo-bis(isobutyronitrile) (AIBN 0.3 mg) were respectively added to separate flasks and the solvent was evaporated from the flasks to leave each residue as a thin film. Each flask was flushed with oxygen, sealed and stored in darkness. The flasks containing 30% AIBN and 5% α -tocopherol **15** were stored for 60 hours prior to analysis whereas the flasks with no additive were stored for 70 hours and for 7 days. For analysis by HPLC, each sample was dissolved in mobile phase (acetonitrile: 30 mM phosphoric acid 80 : 20) and 10% of the sample was analysed (flow rate: 0.6 ml / min; column 3 μ m ODS silica, RI detector). The observed retention times were: lauric acid 8.0 minutes, arachidonic acid **3** 12.7 minutes, the 21:3(n-6) β -oxa compound **83** 13.7 minutes. The results are summarised in Table 3.1.

Competitive autoxidation assay: the 21:3(n-3) β -oxa compound **84 and arachidonic acid **3****

A stock solution was prepared by dissolving arachidonic acid **3** (10 mg), the 21:3(n-3) β -oxa compound **84** (8mg) and lauric acid (6 mg) in dichloromethane (1 ml). Stock solution (100 μ l) was added to three round bottomed flasks. Dichloromethane (5 ml) was added to each flask, α -tocopherol **15** (0.1 mg) and azo-bis(isobutyronitrile) (AIBN 0.1 mg) were respectively added to separate flasks and the solvent was evaporated from the flasks to leave each residue as a thin film. Each flask was flushed with oxygen, sealed and stored in darkness. The flasks containing 10% AIBN and 5% α -tocopherol **15** were stored for 60 hours prior to analysis whereas the flasks with no additive were stored for 70 hours and for 7 days. For analysis under the conditions described above, each sample was dissolved in mobile phase (acetonitrile : 30 mM phosphoric acid, 80 : 20) and 10% of the sample was analysed by HPLC with RI detection. The observed retention times were: lauric acid 8.1 minutes, arachidonic acid **3** 12.9 minutes, the 21:3(n-3) β -oxa compound **84** 13.8 minutes. The results are summarised in Table 3.2.

Competitive autoxidation assay: the 23:4(n-6) β -thia compound **85 and arachidonic acid **3****

A stock solution was prepared by dissolving arachidonic acid **3** (10 mg), the 23:4(n-6) β -thia compound **85** (8 mg) and lauric acid (6 mg) in dichloromethane (1 ml). Stock solution (100 μ l) was added to three round bottomed flasks. Dichloromethane (5 ml) was added to each flask, α -tocopherol **15** (0.1 mg) and AIBN (0.2 mg) were respectively added to separate flasks and the solvent was evaporated from the flasks to leave the residues as thin films. Each flask was flushed with oxygen, sealed and stored in darkness. The flasks containing 20% AIBN and 5% α -tocopherol **15** were stored for 60 hours prior to analysis whereas the flasks with no additive were stored for 70 hours and for 7 days. For analysis under the conditions described above, each sample was

dissolved in mobile phase and 10% of the sample was analysed by HPLC with RI detection. The observed retention times were: lauric acid 8.0 minutes, arachidonic acid **3** 12.7 minutes, the 23:4(n-6) β -thia compound **85** 18.5 minutes. The results are summarised in Table 3.3.

Competitive autoxidation assay: the 24:4(n-6) γ -thia compound **86 and arachidonic acid **3****

A stock solution was prepared by dissolving arachidonic acid **3** (10 mg), the 24:4(n-6) γ -thia compound **86** (8 mg) and lauric acid (6 mg) in dichloromethane (1 ml). Stock solution (100 μ l) was added to three round bottomed flasks. Dichloromethane (5 ml) was added to each flask, α -tocopherol (0.1 mg) and AIBN (0.2 mg) were respectively added to separate flasks and the solvent was evaporated from the flasks to leave each residue as a thin film. Each flask was flushed with oxygen, sealed and stored in darkness. The flasks containing 20% AIBN and 5% α -tocopherol **15** were stored for 60 hours prior to analysis whereas the flasks with no additive were stored for 70 hours and for 7 days. For analysis under the conditions described above, each sample was dissolved in mobile phase and 10% of the sample was analysed by HPLC with RI detection. The observed retention times were: lauric acid 8.1 minutes, arachidonic acid **3** 12.9 minutes, the 24:4(n-6) γ -thia compound **86** 20.0 minutes. The results are summarised in Table 3.4.

Consumption of arachidonic acid **3 (1 mM) during incubation with soybean 15-LO (18 μ g / ml)**

Arachidonic acid **3** (1.75 mg) and standard (lauric acid, 1.25 mg) were added to the reaction flask in dichloromethane, the solvent was evaporated under a stream of nitrogen and the residue was suspended in buffer (1ml, pH 9.0, 0.1 M KH_2PO_4) by vigorous stirring. An aliquot (100 μ l) of the mixture was taken for HPLC analysis. Each aliquot

was extracted into dichloromethane, the dichloromethane was evaporated, the residue was resuspended in HPLC mobile phase and analysed by HPLC. 15-LO (3.6 ml, 18 $\mu\text{g} / \text{ml}$) was added to the mixture and an aliquot (700 μl) for HPLC analysis was taken after 12 minutes. This aliquot contained 30% less arachidonic acid **3** than the aliquot taken prior to incubation with 15-LO.

Consumption of arachidonic acid **3 (3 mM) during incubation with potato 5-LO (20% v/v)**

Arachidonic acid **3** (4.2 mg) and standard (lauric acid, 3.0 mg) were added to the reaction flask in dichloromethane, the solvent was evaporated under a stream of nitrogen and the residue was suspended in buffer (4.0 ml, pH 6.0, 0.04 M KH_2PO_4) by vigorous stirring. An aliquot (0.5 ml) of the mixture was taken for HPLC analysis. Each aliquot was extracted into dichloromethane, the dichloromethane was evaporated, the residue was resuspended in HPLC mobile phase and analysed by HPLC. Potato 5-LO extract (0.9 ml) was added to the mixture and an aliquot (0.5 ml) for HPLC analysis was taken after 16 minutes. This aliquot contained 30% less arachidonic acid **3** than the aliquot taken prior to incubation with 5-LO.

Consumption of arachidonic acid **3 (90 μM) during incubation with soybean 15-LO (2 $\mu\text{g} / \text{ml}$) for 8 minutes**

Arachidonic acid **3** (750 μg) and standard (lauric acid, 880 μg) were suspended in buffer (27.2 ml, pH 9.0, 0.1 M KH_2PO_4) by vigorous stirring. An aliquot (9.1 ml) of the mixture was taken for HPLC analysis. Each aliquot was extracted into dichloromethane, the dichloromethane was evaporated, the residue was resuspended in HPLC mobile phase and analysed by HPLC. 15-LO (188 μl , 200 $\mu\text{g} / \text{ml}$) was added to the mixture and an

aliquot (9 ml) for HPLC analysis was taken after 8 minutes. This aliquot contained 52% of the arachidonic acid **3** present in the aliquot taken prior to incubation with 5-LO.

Consumption of arachidonic acid **3 (90 μ M) during incubation with soybean 15-LO (4 μ g / ml) for 8 minutes**

Arachidonic acid **3** (750 μ g, 2.5 μ mol) and standard (lauric acid, 880 μ g, 4.4 μ mol) were added to the reaction flask in dichloromethane, the solvent was evaporated under a stream of nitrogen and the residue was suspended in buffer (26.9 ml, pH 9.0, 0.1 M KH_2PO_4) by vigorous stirring. An aliquot (9.0 ml) of the mixture was taken for HPLC analysis. Each aliquot was extracted into dichloromethane, the dichloromethane was evaporated, the residue was resuspended in HPLC mobile phase and analysed by HPLC. 15-LO (375 μ l, 200 μ g / ml) was added to the mixture and an aliquot (9 ml) for HPLC analysis was taken after 8 minutes. This aliquot contained less than 5% of the arachidonic acid **3** present in the aliquot taken prior to incubation with 15-LO.

Consumption of arachidonic acid **3 and the 21:3(n-3) β -oxa compound **84** by potato 5-LO**

Arachidonic acid **3** (750 μ g, 2.5 μ mol, 1 mass equivalent) and internal standard lauric acid (750 μ g, 3.7 μ mol) were added to each of six 25 ml conical flasks from stock solutions in dichloromethane. The 21:3(n-3) β -oxa compound **84** (0, 0.25, 0.5, 1, 2 or 4 mass equivalents) was added to the flasks from stock solution in dichloromethane. The dichloromethane was evaporated under nitrogen and the residues were suspended in buffer (22.8 ml) with vigorous stirring at room temperature. An aliquot (7.6 ml) was removed as a sample representative of initial conditions. Potato 5-LO extract (3.1 ml) was added to the stirred solutions and aliquots of the solutions were removed for analysis after 8 and 16 minutes. Immediately after removal, these aliquots were added to

dichloromethane (10 ml), acidified (2 drops concentrated HCl) and stirred for an hour. The dichloromethane was separated and evaporated under nitrogen. The residues were resuspended in HPLC solvent (150 μ l) and sonicated before being transferred to autosampler vials and analysed by reverse phase HPLC with RI detection under the conditions outlined above. The observed retention times were: lauric acid 9.1 minutes, arachidonic acid **3** 16.0 minutes, the 21:3(n-3) β -oxa compound **84** 14.9 minutes. The results are presented in Figs 3.2 and 3.3.

Consumption of arachidonic acid **3 and the 21:3(n-3) β -oxa compound **84** by soybean 15-LO**

Arachidonic acid **3** (750 μ g, 2.5 μ mol, 1 mass equivalent) and internal standard lauric acid (750 μ g, 3.7 μ mol) were added to each of six 25 ml conical flasks from stock solutions in dichloromethane. The 21:3(n-3) β -oxa compound **84** (0, 0.25, 0.5, 1, 2 or 4 mass equivalents) was added to the flasks from stock solution in dichloromethane. The dichloromethane was evaporated under nitrogen and the residues were suspended in buffer (27.1 ml) with vigorous stirring at room temperature. An aliquot (9.0 ml) was removed as a sample representative of initial conditions. Soybean 15-LO (35 μ g in 190 μ l) was added to the stirred solutions and aliquots (9 ml) of the solutions were removed for analysis after 8 and 16 minutes. Immediately after removal, these aliquots were added to dichloromethane (10 ml), acidified (2 drops concentrated HCl) and stirred for an hour. The dichloromethane was separated and evaporated under nitrogen. The residues were resuspended in HPLC solvent (150 μ l) and sonicated before being transferred to autosampler vials and analysed by reverse phase HPLC with RI detection under the conditions outlined above. The observed retention times were: lauric acid 8.4 minutes, arachidonic acid **3** 14.1 minutes, the 21:3(n-3) β -oxa compound **84** 13.3 minutes. The results are summarised in Figs 3.4 and 3.5.

Consumption of arachidonic acid **3 and the 21:3(n-6) β -oxa compound **83** by soybean 15-LO**

Arachidonic acid **3** (600 μg , 2.0 μmol , 1 mass equivalent) and internal standard lauric acid **104** (600 μg , 3.0 μmol) were added to each of six 25 ml conical flasks from stock solutions in dichloromethane. The 21:3(n-6) β -oxa compound **83** (0, 0.25, 0.5, 1, 2 or 4 mass equivalents) was added to the flasks from stock solution in dichloromethane. The dichloromethane was evaporated under nitrogen and the residues were suspended in buffer (21.7 ml, pH = 9.0, 0.1 M KH_2PO_4) with vigorous stirring at room temperature. An aliquot (7.2 ml) was removed as a sample representative of initial conditions. Soybean 15-LO (190 μl , 184 μg / ml) was added to the stirred solutions and aliquots (9 ml) of the solutions were removed for analysis after 8 and 16 minutes. Immediately after removal, these aliquots were added to dichloromethane (10 ml), acidified (2 drops concentrated HCl) and stirred for an hour. The dichloromethane layer was separated and evaporated under nitrogen. The residues were resuspended in HPLC solvent (150 μl) and sonicated before being transferred to autosampler vials and analysed by reverse phase HPLC with RI detection under the conditions outlined above. The observed retention times were: lauric acid 8.5 minutes, arachidonic acid **3** 14.3 minutes, the 21:3(n-6) β -oxa compound **83** 13.5 minutes. The results are summarised in Figs 3.6 and 3.7.

Consumption of arachidonic acid **3 and the 24:4(n-6) γ -thia compound **86** by potato 5-LO**

Arachidonic acid **3** (750 μg , 2.5 μmol , 1 mass equivalent) and internal standard lauric acid (750 μg , 3.8 μmol) were added to each of six 25 ml conical flasks from stock solutions in dichloromethane. The 24:4(n-6) γ -thia compound **86** (0, 0.25, 0.5, 1, 2 or 4 mass equivalents) was added to the flasks from stock solution in dichloromethane. The dichloromethane was evaporated under nitrogen and the residues were suspended in buffer (26 ml) with vigorous stirring at room temperature. An aliquot (8.7 ml) was

removed as a sample representative of initial conditions. Potato 5-LO extract (1.0 ml) was added to the stirred solutions and aliquots of the solutions were removed for analysis after 8 and 16 minutes. Immediately after removal, these aliquots were added to dichloromethane (10 ml), acidified (2 drops concentrated HCl) and stirred for an hour. The dichloromethane was separated and evaporated under nitrogen. The residues were resuspended in HPLC solvent (150 μ l) and sonicated before being transferred to autosampler vials and analysed by reverse phase HPLC with RI detection as outlined above. The observed retention times were: lauric acid 7.7 minutes, arachidonic acid **3** 12.4 minutes, the 24:4(n-6) γ -thia compound **86** 20.0 minutes. The results are summarised in Figs 3.8 and 3.9.

Consumption of arachidonic acid **3 and the 24:4(n-6) γ -thia compound **86** by soybean 15-LO**

Arachidonic acid **3** (750 μ g, 2.5 μ mol, 1 mass equivalent) and internal standard lauric acid (750 μ g, 3.8 μ mol) were added to each of six 25 ml conical flasks from stock solutions in dichloromethane. The 24:4(n-6) γ -thia compound **86** (0, 0.25, 0.5, 1, 2 or 4 mass equivalents) was added to each of the flasks from stock solution in dichloromethane. The dichloromethane was evaporated under nitrogen and the residues were suspended in buffer (26.3 ml) with vigorous stirring at room temperature. An aliquot (8.75 ml) was removed as a sample representative of initial conditions. Soybean 15-LO (150 μ g in 0.75 ml) was added to the stirred solutions and aliquots of the solutions were removed for analysis after 8 and 16 minutes. Immediately after removal, these aliquots were added to dichloromethane (10 ml), acidified (2 drops concentrated HCl) and stirred for an hour. The dichloromethane was separated and evaporated under nitrogen. The residues were resuspended in HPLC solvent (150 μ l) and sonicated before being transferred to autosampler vials and analysed by reverse phase HPLC with RI detection under the conditions outlined above. The observed retention times were: lauric

acid 7.6 minutes, arachidonic acid **3** 11.9 minutes, the 24:4(n-6) γ -thia compound **86** 18.5 minutes. The results are summarised in Figs 3.10 and 3.11.

Experimental for Results and Discussion: Chapter 3

Monitoring the formation of oxidation products containing the conjugated *E,Z*-diene moiety by UV spectroscopy at 234 nm

Stock solution concentrations for each of the fatty acids **3**, **5** and **83-89** in dichloromethane and the volumes of each stock solution used to prepare solutions (20 and 50 μ M) for the spectroscopy experiments were as follows:

The arachidonic acid **3** (0.66 mM) stock solution contained 20 mg / 100 ml. Hence, the residue from 190 μ l and 475 μ l of stock solution was used in a final volume of 2.5 ml buffer to prepare 20 μ M and 50 μ M samples for incubation with 15-LO and 5-LO, respectively.

The docosahexaenoic acid **5** (3.23 mM) stock solution contained 10.6 mg / 10 ml. Hence, the residue from 15.5 μ l and 38.8 μ l of stock solution was used in a final volume of 2.5 ml buffer to prepare 20 μ M and 50 μ M samples for incubation with 15-LO and 5-LO, respectively.

The (n-6)- β -oxa compound **83** (3.35 mM) stock solution contained 5.4 mg / 5 ml. Hence, the residue from 14.9 μ l and 37.3 μ l of stock solution was used in a final volume of 2.5 ml buffer to prepare 20 μ M and 50 μ M samples for incubation with 15-LO and 5-LO, respectively.

The (n-3)- β -oxa compound **84** (3.35 mM) stock solution contained 5.4 mg / 5 ml. Hence, the residue from 14.9 μ l and 37.3 μ l of stock solution was used in a final volume

of 2.5 ml buffer to prepare 20 μ M and 50 μ M samples for incubation with 15-LO and 5-LO, respectively.

The (n-6)- β -thia compound **85** (3.56 mM) stock solution contained 6.5 mg / 5 ml. Hence, the residue from 14.0 μ l and 35 μ l of stock solution was used in a final volume of 2.5 ml buffer to prepare 20 μ M and 50 μ M samples for incubation with 15-LO and 5-LO, respectively.

The (n-6)- γ -thia compound **86** (2.59 mM) stock solution contained 4.9 mg / 5 ml. Hence, the residue from 19.3 μ l and 48.3 μ l of stock solution was used in a final volume of 2.5 ml buffer to prepare 20 μ M and 50 μ M samples for incubation with 15-LO and 5-LO, respectively.

The α -branched- β -thia diacid **87** (2.75 mM) stock solution contained 5.8 mg / 5 ml. Hence, the residue from 18.2 μ l and 45.5 μ l of stock solution was used in a final volume of 2.5 ml buffer to prepare 20 μ M and 50 μ M samples for incubation with 15-LO and 5-LO, respectively.

The (n-6)-amide **88** (1.63 mM) stock solution contained 5.9 mg / 10 ml. Hence, the residue from 30.6 μ l and 76.5 μ l of stock solution was used in 2.5 ml buffer to prepare 20 μ M and 50 μ M samples for incubation with 15-LO and 5-LO, respectively.

The α -branched amide **89** (1.39 mM) stock solution contained 5.0 mg / 10 ml. Hence, the residue from 42.0 μ l and 105.0 μ l of stock solution was used in 2.5 ml buffer to prepare 20 μ M and 50 μ M samples for incubation with 15-LO and 5-LO, respectively.

Arachidonic acid 3 consumption using soybean 15-LO (0.4-1.6 μg / ml)

Arachidonic acid **3** (15 μg , 50 nmol) was added to three quartz glass cuvettes (3 ml) from stock solution in dichloromethane to give a final concentration of 20 μM in 2.5 ml buffer. The solvent was evaporated under nitrogen and the residue was dissolved in various volumes of buffer solution (pH 9.0, 0.1 M KH_2PO_4) to give sample A (2.44 ml buffer), sample B (2.47 ml buffer) and sample C (2.485 ml buffer). Soybean 15-LO was added to samples A (60 μl , 4 μg), B (30 μl , 2 μg) and C (15 μl , 1 μg) and the change in absorbance at 234 nm was monitored for 10 minutes in each case. The results are shown in Fig 4.1 and are summarised in Table 4.1.

Consumption of the fatty acids 3, 5 and 83-89 using soybean 15-LO (0.8 μg / ml)

The fatty acid **3**, **5** or **83-89** (50 nmol) was added to a quartz cuvette (3 ml) from stock solution in dichloromethane (as outlined above) to give a final concentration of 20 μM in 2.5 ml buffer. The solvent was evaporated under nitrogen and the residue was dissolved in buffer solution (2.47 ml, pH 9.0, 0.1 M KH_2PO_4). Soybean 15-LO (2 μg in 30 μl buffer) was added and the change in absorption (234 nm) was monitored for 10 minutes. The results are shown in Figs 4.2-4.3 and are summarised in Table 4.2. In Fig 4.4 the result is shown for the experiment in which the volume of 15-LO stock solution was doubled to 60 μl in 2.44 ml of buffer with the branched- β -thia diacid **87** (20 μM).

The effect of Tween[®] 20 on the oxidation of arachidonic acid 3 using potato 5-LO⁶⁰

Arachidonic acid **3** (38 μg in 475 μl) was added to quartz glass cuvettes (3 ml) from stock solution in dichloromethane to give a final concentration of 50 μM in 2.5 ml buffer.

The solvent was evaporated under nitrogen and the residue was dissolved in buffer solution (2.45 ml, pH 5.5, 0.1 M KH_2PO_4) containing a range of concentrations (0, 0.0005%, 0.005%, 0.05% and 0.5% as volume of Tween[®] 20 per volume of buffer) of Tween[®] 20 (sorbitan monolaurate). A second solution of the sample with 0.005% Tween[®] 20 was saturated with oxygen by bubbling oxygen gas into the cuvette for 30 seconds followed by vigorous shaking of the sealed solution for 30 seconds. Crude potato 5-LO extract (50 μl) was added and the change in absorption (234 nm) was monitored for 5 minutes in each case. The results are summarised in Table 4.3.

The change in absorbance over time during incubation of the PUFAs 3 with several concentrations of potato 5-LO

Arachidonic acid **3** (38 μg in 475 μl) was added to three quartz glass cuvettes (3 ml) from stock solution in dichloromethane to give a final concentration of 50 μM in 2.5 ml buffer. The solvent was evaporated under nitrogen and the residues were dissolved in 2.40, 2.45 and 2.475 ml of buffer solution (pH 5.5, 0.005% Tween[®] 20, 0.1 M KH_2PO_4). The solution was saturated with oxygen by bubbling oxygen gas into the cuvette for 30 seconds followed by vigorous shaking of the sealed solution for 30 seconds. Crude potato 5-LO extract (100, 50 and 25 μl) was added to the cuvettes containing 2.40, 2.45 and 2.475 ml of buffer, respectively, and the change in absorption at 234 nm was monitored for 5 minutes. The results are shown in Fig 4.6.

The change in absorbance over time during incubation of the PUFAs 3, 5 or 83-89 with potato 5-LO

PUFA **3**, **5** or **83-89** was added from stock solution in dichloromethane to give a final concentration of 50 μM in 2.5 ml buffer. The solvent was evaporated under nitrogen and the residue was dissolved in buffer solution (2.45 ml, pH 5.5, 0.005% Tween[®] 20, 0.1

M KH_2PO_4). The solution was saturated with oxygen by bubbling oxygen gas into the cuvette for 30 seconds followed by vigorous shaking of the sealed solution for 30 seconds. Crude potato 5-LO extract (50 μl) was added and the change in absorption (234 nm) was monitored for 5 minutes. The results are shown in Fig 4.7 for arachidonic acid **3**, the unbranched amide **88**, the γ -thia compound **86** and the β -oxa compound **83**.

Experimental for Results and Discussion: Chapter 4

Products of soybean 15-LO catalysed oxidation

(*Z,Z,E*)-(13-Hydroxyoctadeca-6,9,11-trienyloxy)acetic acid **96**

(*Z,Z,Z*)-(octadeca-6,9,12-trienyloxy)acetic acid **83** (50 mg, 155 μmol) was weighed into a round bottom flask and dissolved in phosphate buffer (0.1 M, pH = 9.0, 200 ml, 30 °C). Soybean lipoxygenase (15 mg) was added in phosphate buffer (5 ml) and oxygen was bubbled through the stirred solution for 10 minutes. The reaction was terminated by the addition of triphenylphosphine (50 mg, 1.1 equiv.) in cold dichloromethane (200 ml) followed by hydrochloric acid (0.2 N, 20 ml). The solution was stirred on ice for 45 minutes. The crude hydroxy β -oxa fatty acid **96** was extracted with dichloromethane and the organic extracts were combined and concentrated. By analytical HPLC (hexane : isopropanol : acetic acid, 960 : 40 : 1; flow rate 0.6 ml / min) with detection of the conjugated diene moiety at 234 nm, a single main product had formed with a retention time of 15 minutes as shown in Fig 5.1. (*Z,Z,E*)-(13-Hydroxyoctadeca-6,9,11-trienyloxy)acetic acid **96** was isolated following chromatography on silica (ether : hexane : acetic acid, 80 : 20 : 0.1) in a 23 % yield (12 mg, 36 μmol).

^1H nmr (300 MHz, CDCl_3) δ 0.89 (3H, t, 6.8 Hz, C18'-H₃), 1.25-1.45 (10H, m) 1.59-1.73 (2H, m, C2'-H₂), 2.01-2.12 (4H, m, C5'-H₂, C14'-H₂), 2.81 (2H, t, C8'-H₂), 3.56 (2H, dt, C1'-H₂), 4.10 (2H, s, C2-H₂), 4.20 (1H, dt, 6.0, 6.6 Hz, C13'-H),

5.29-5.45 (3H, m, C6'-H, C7'-H, C9'-H), 5.70 (1H, dd, 6.7, 15.2 Hz, C12'-H), 5.99 (1H, dd, 10.9 Hz, C10'-H), 6.55 (1H, dd, 10.9, 15.1 Hz, C11'-H) as shown in Fig 5.2.

Negative ion electrospray MS / MS, daughter ions of molecular anion: 337, 319, 237, 75 as shown in Fig 5.3. Positive ion electrospray HRMS $[C_{20}H_{34}O_4 + Na]^+$ calculated 361.2355, found 361.2361.

(Z,E,Z)-(13-Hydroxyoctadeca-9,11,15-trienyloxy)acetic acid 97

(Z,Z,Z)-(Octadeca-9,12,15-trienyloxy)acetic acid **84** (50 mg, 155 μ mol) was weighed into a round bottom flask and dissolved in phosphate buffer (0.1 M, pH = 9.0, 45 ml, 30 °C). Soybean lipoxygenase (8 mg) was added in phosphate buffer (5 ml) and oxygen was bubbled through the stirred solution for 10 minutes. The reaction was terminated by the addition of triphenylphosphine (50 mg, 1.1 equiv.) in cold dichloromethane (50 ml) followed by hydrochloric acid (0.2 N, 20 ml). The solution was stirred on ice for 20 minutes. The crude hydroxy β -oxa fatty acid was extracted with dichloromethane and the organic extracts were combined and concentrated. By analytical HPLC (hexane : isopropanol : acetic acid, 940 : 60 : 1; flow rate 0.6 ml / min) with detection of the conjugated diene moiety at 234 nm, a single main product had formed with a retention time of 12 minutes as shown in Fig 5.15. (Z,E,Z)-13-(Hydroxyoctadeca-9,11,15-trienyloxy)acetic acid (15 mg, 44 μ mol, 29 %) was isolated following chromatography on silica, eluting with ethyl acetate : hexane : acetic acid (80 : 20 : 0.1).

1H nmr (300 MHz, $CDCl_3$) δ 0.95 (3H, t, 7.6 Hz, C18'-H₃), 1.17-1.40 (10H, m), 1.48-1.63 (2H, m), 1.99-2.12 (2H, m), 2.13-2.22 (2H, m), 2.28-2.37 (2H, m), 3.34-3.50 (2H, m, C1'-H₂), 3.80-3.92 (2H, s, C2-H₂), 4.14-4.20 (1H, dt, C13'-H), 5.30-5.60 (3H, m, C9'-H, C15'-H, C16'-H), 5.67 (1H, dd, C12'-H), 5.95 (1H, t, C10'-H), 6.49 (1H, dd, C11'-H)

^{13}C nmr (CDCl_3) δ 14.1 ($\text{C18}'$), 20.6, 25.6, 27.6, 28.9, 29.0, 29.1, 29.2, 29.3, 29.6, 35.1($\text{C1}'$), 53.3, 67.6, 72.0($\text{C13}'$), 123.5, 125.8, 127.6, 132.9, 134.7, 135.2

Negative ion electrospray MS/MS, daughter ions of molecular anion: 337, 319, 268, 267, 240, 239, 75 as shown in Fig 5.4. Positive ion electrospray HRMS [$\text{C}_{20}\text{H}_{34}\text{O}_4 + \text{Na}]^+$ calculated 361.2355, found 361.2345.

(Z,Z,Z,E)-(15-Hydroxyeicosa-5,8,11,13-tetraenylthio)acetic acid 98

(Z,Z,Z,Z)-(Eicosa-5,8,11,14-tetraenylthio)acetic acid **85** (43 mg, 118 μmol) was weighed into a round bottom flask and dissolved in phosphate buffer (0.1 M, pH = 9.0, 300 ml, 30 °C). Soybean lipoxygenase (22 mg) was added in phosphate buffer (5 ml) and oxygen was bubbled through the stirred solution for 10 minutes. The reaction was terminated by the addition of sodium borohydride (23 mg) in cold ethanol (10 ml) and the solution was stirred on ice for 45 minutes. Hydrochloric acid (0.2 N, 20 ml) was added and the crude hydroxy β -thia fatty acid **98** was extracted with dichloromethane (3 x 100ml) and the organic extracts were combined and concentrated. By analytical HPLC (hexane : isopropanol : acetic acid, 940 : 60 : 1; flow rate 1.0 ml / min) with detection of the conjugated diene moiety at 234 nm, a single main product had formed with a retention time of 10 minutes. The product was purified by chromatography on silica (ether : hexane : acetic acid, 60 : 40 : 2) to yield the (Z,Z,Z,E)-15-(Hydroxyeicosa-5,8,11,13-tetraenylthio)acetic acid **98** (8 mg, 21 μmol , 18%).

^1H nmr (300 MHz, CDCl_3) δ 0.82 (3H, t, 6.9 Hz, $\text{C20}'\text{-H}_3$), 1.15-1.32 (8H, m), 1.32-1.63 (4H, m), 1.92-2.06 (2H, m), 2.50-2.65 (2H, m), 2.65-2.84 (2H, m), 2.84-2.94 (2H, m), 3.17 (2H, s, C2-H_2), 4.10-4.20 (1H, m, $\text{C15}'\text{-H}$), 5.23-5.40 (5H, m, $\text{C5}'\text{-H}$, $\text{C6}'\text{-H}$, $\text{C8}'\text{-H}$, $\text{C9}'\text{-H}$, $\text{C11}'\text{-H}$), 5.60 (1H, dd, 6.0, 15.0 Hz, $\text{C14}'\text{-H}$), 5.93 (1H, t, 11.7 Hz, $\text{C12}'\text{-H}$), 6.48 (1H, dd, 11.1, 15.3 Hz, $\text{C13}'\text{-H}$)

^{13}C nmr (CDCl_3) δ 14.0 ($\text{C20}'$), 22.5, 24.9, 25.6, 26.0, 26.5, 28.4, 29.6, 30.2, 31.6, 37.2, 58.6, 72.8 ($\text{C13}'$), 125.4, 127.3, 128.0, 128.5, 129.5, 130.1, 130.3, 135.8

Negative ion electrospray MS/MS, daughter ions of molecular anion: 379, 361, 335, 317, 235, 113 as shown in Fig 5.5. Positive ion electrospray HRMS $[C_{22}H_{36}SO_3 + Na]^+$ calculated 403.2283, found 403.2290.

3-[(Z,Z,Z,E)-(15-Hydroxyeicosa-5,8,11,13-tetraenylthio)]propionic acid **99**

3-[(Z,Z,Z,Z)-(eicosa-5,8,11,14-tetraenylthio)]propionic acid **86** (10 mg, 26 μ mol) was weighed into a round bottom flask and dissolved in phosphate buffer (0.1 M, pH = 9.0, 100 ml, 30 °C). Soybean lipoxygenase (5 mg) was added in phosphate buffer (5 ml) and oxygen was bubbled through the stirred solution for 10 minutes. The reaction was terminated by the addition of triphenylphosphine (8 mg, 1.1 equiv.) in cold dichloromethane (50 ml) followed by hydrochloric acid (0.2 N, 20 ml). The solution was stirred on ice for 20 minutes. The crude hydroxy γ -thia fatty acid **99** was extracted with dichloromethane (2 x 100 ml) and the organic extracts were combined and concentrated. By analytical HPLC (hexane : isopropanol : acetic acid, 940 : 60 : 1; flow rate 1 ml / min) with detection of the conjugated diene moiety at 234 nm, a single main product had formed. 3-[(Z,Z,Z,E)-15-(Hydroxyeicosa-5,8,11,13-tetraenylthio)]propionic acid **99** (2.5 mg, 6.4 μ mol, 24%) was isolated following chromatography on silica (ethyl acetate : hexane : acetic acid, 80 : 20 : 0.1).

1H nmr (300 MHz, $CDCl_3$) δ 0.82 (3H, m, C20'-H₃), 1.13-1.36 (8H, m), 1.36-1.53 (4H, m), 1.53-1.75 (2H, m), 1.95-2.11 (2H, m), 2.63-2.79 (4H, m), 2.79-3.08 (2H, m), 4.03-4.17 (1H, m, C13'-H), 5.24-5.35 (5H, m), 5.63 (1H, dd, 15.3, 6.6 Hz, C14'-H), 5.93 (1H, t, 10.7 Hz, C12'-H), 6.46 (1H, dd, 10.9, 14.8 Hz, C13'-H)

Negative ion electrospray MS/MS, daughter ions of molecular anion: 393, 375, 321, 303, 257, 221, 175, 113, 71 as shown in Fig 5.6. Positive ion electrospray HRMS $[C_{22}H_{38}SO_3 + Na]^+$ calculated 417.2439, found 417.2465.

[Z,Z,Z,E]-(15-Hydroxyeicosa-5,8,11,13-tetraenylsulfoxy)succinic acid
100

[Z,Z,Z,Z]-(Eicosa-5,8,11,14-tetraenylthio)succinic acid **87** (42 mg, 100 μ mol) was weighed into a round bottom flask and dissolved in phosphate buffer (0.1 M, pH = 9.0, 300 ml, 30 °C). Soybean lipoxygenase (20 mg) was added in phosphate buffer (5 ml) and oxygen was bubbled through the stirred solution for 10 minutes. The reaction was terminated by the addition of sodium borohydride (15 mg) in ethanol (5 ml) and stirred on ice for 45 minutes. Hydrochloric acid (0.2 N, 20 ml) was added, the crude hydroxy-fatty acid **100** was extracted with dichloromethane (2 x 100 ml) and the organic extracts were combined and concentrated. By analytical HPLC (hexane : isopropanol : acetic acid, 900 : 100 : 1; flow rate 1.0 ml / min) with detection of the conjugated diene moiety at 234 nm, a single main product had formed with a retention time of 29 minutes. [Z,Z,Z,E]-(15-Hydroxyeicosa-5,8,11,13-tetraenylsulfoxy)succinic acid **100** was purified by chromatography on silica (ether : ethyl acetate : acetic acid, 50 : 50 : 10) to yield 16 mg (35 μ mol, 35 % yield).

^1H nmr (300 MHz, CDCl_3) δ 0.81 (3H, m, C20'-H₃), 1.10-1.35 (8H, m), 1.35-1.60 (4H, m), 1.60-1.87 (2H, m), 1.87-2.16 (4H, m), 2.75 (2H, s), 2.89 (2H, s), 3.96 (1H, s), 4.12 (1H, m, C15'-H), 5.20-5.38 (5H, m), 5.61 (1H, dd, 6.5, 14.8 Hz, C14'-H), 5.92 (1H, t, 10.9 Hz, C12'-H), 6.45 (1H, dd, 11.2, 15.1 Hz, C13'-H)

^{13}C nmr (CDCl_3) δ 14.5 (C20'), 21.1, 23.0, 23.1, 23.2, 25.5, 26.2, 26.6, 27.2, 28.9, 32.2, 37.6, 73.3(C13'), 126.0, 128.1, 128.5, 129.0, 129.6, 130.7, 136.5

Negative ion electrospray MS/MS, daughter ions of molecular anion: 453, 409, 319, 301, 255, 219, 173, 120, 71, 59 as shown in Fig 5.7. Positive ion electrospray HRMS $[\text{C}_{24}\text{H}_{38}\text{SO}_6 + \text{Na}]^+$ calculated 477.2287, found 477.2282.

***N*-(*Z,Z,Z,E*)-(15-Hydroxyeicosa-5,8,11,13-tetraenyl)glycine 101**

N-(*Z,Z,Z,Z*)-(eicosa-5,8,11,14-tetraenyl)glycine **88** (25 mg, 60 μ mol) was weighed into a round bottom flask and dissolved in phosphate buffer (0.1 M, pH = 9.0, 250 ml, 30 °C). Soybean lipoxygenase (15 mg) was added in phosphate buffer (5 ml) and oxygen was bubbled through the stirred solution for 20 minutes. The reaction was terminated by the addition of sodium borohydride (10 mg) in ethanol (5 ml) and stirred on ice for 45 minutes. Hydrochloric acid (0.2 N, 20 ml) was added, the crude hydroxy-fatty acid **100** was extracted with dichloromethane (2 x 100 ml) and the organic extracts were combined and concentrated. By analytical HPLC (hexane : isopropanol : acetic acid, 820 : 180 : 1; flow rate 0.6 ml / min) with detection of the conjugated diene moiety at 234 nm, a single main product had formed with a retention time of 15 minutes. *N*-(*Z,Z,Z,E*)-(15-Hydroxyeicosa-5,8,11,13-tetraenyl)glycine **101** was purified by chromatography on silica (ether : ethyl acetate : acetic acid, 90 : 10 : 5) to yield 9 mg (24 μ mol, 40 % yield).

¹H nmr (300 MHz, CDCl₃) δ 0.81 (3H, m, C20'-H₃), 0.98-1.25 (8H, m), 1.62-1.72 (2H, m), 1.97-2.10 (2H, m), 2.10-2.23 (2H, m), 2.70-2.77 (2H, m), 2.85-2.95 (2H, m), 3.98 (2H, s, C2-H₂), 4.14-4.22 (1H, m, C13'-H), 5.23-5.39 (5H, m), 5.63 (1H, dd, 6.0, 15.6 Hz, C14'-H), 5.93 (1H, t, 11.1 Hz, C12'-H), 6.22 (1H, s, NH), 6.51 (1H, dd, 11.2, 15.2 Hz, C13'-H)

¹³C nmr (CDCl₃) δ 13.9 (C20'), 22.4, 22.6, 25.0, 25.7, 26.1, 26.2, 29.3, 29.6, 35.2, 37.2, 41.3, 72.7 (C13'), 125.3, 127.4, 127.8, 128.4, 129.0, 130.3, 135.8, 173.8 (C=O), 172.0 (C=O)

Negative ion electrospray MS/MS, daughter ions of molecular anion: 376, 358, 276, 175, 74 as shown in Fig 5.8. Positive ion electrospray HRMS [C₂₂H₃₅NO₄ + Na]⁺ calculated 400.2464, found 400.2468.

N*-(*Z,Z,Z,E*)-(15-Hydroxyeicosa-5,8,11,13-tetraenyl)aspartic acid **102*

[5'*Z*,8'*Z*,11'*Z*,14'*Z*]-eicosatetraenylaspartic acid **88** (36 mg, 86 μ mol) was weighed into a round bottom flask and dissolved in phosphate buffer (0.1 M, pH = 9.0, 300 ml, 30 °C). Soybean lipoxygenase (20 mg) was added in phosphate buffer (5 ml) and oxygen was bubbled through the stirred solution for 20 minutes. The reaction was terminated by the addition of sodium borohydride (10 mg) in ethanol (5 ml) and stirred on ice for 45 minutes. Hydrochloric acid (0.2 N, 20 ml) was added, the crude hydroxy-fatty acid **102** was extracted with dichloromethane (2 x 100 ml) and the organic extracts were combined and concentrated. By analytical HPLC (hexane : isopropanol : acetic acid, 800 : 200 : 5; flow rate 0.6 ml / min) with detection of the conjugated diene moiety at 234 nm, a single main product had formed with a retention time of 13 minutes. *N*-(*Z,Z,Z,E*)-(15-Hydroxyeicosa-5,8,11,13-tetraenyl)aspartic acid **102** was purified by chromatography on silica (ethyl acetate : acetic acid, 100 : 5) to yield 6 mg (14 μ mol, 39 % yield).

¹H nmr (300 MHz, CDCl₃) δ 0.81 (3H, m, C20'-H₃), 1.10-1.75 (10H, m), 1.85-2.10 (2H, m), 2.10-2.30 (2H, m), 2.60-3.00 (m, 4H), 4.00-4.20 (1H, m), 4.20-4.32 (1H, m), 5.20-5.40 (5H, m), 5.50-5.75 (1H, m, C14'), 5.85-6.00 (1H, m, C12'), 6.38-6.55 (1H, m, C13')

Negative ion electrospray MS/MS, daughter ions of molecular anion: 434, 390, 319, 300, 257, 115, 88, 71, 58 as shown in Fig 5.9. Positive ion electrospray HRMS [C₂₄H₃₇NO₆ + Na]⁺ calculated 458.2519, found 458.2508.

Products of potato 5-LO catalysed oxidation:

Each of the synthetic PUFAs **83-89** were prepared at a 100 μ M concentration in 2.48 ml buffer (0.04 M, 0.01% Brij58, pH = 6.0, 35 °C). Potato 5-LO (210 μ l) extract was added and the mixtures were incubated for 20 minutes. Sodium borohydride (0.5 mg) was dissolved in the buffer and after 30 minutes the products were extracted into

dichloromethane (2 x 5 ml) following acidification of the buffer. The organic solvent was evaporated using a stream of nitrogen, the residue was resuspended in the HPLC solvent indicated below and the products were separated by HPLC with detection at 234 nm.

The 3-[(*E,Z,Z,Z*)-(5-Hydroxyeicosa-6,8,11,13-tetraenylthio)]propionic acid **103** formed by oxidation of (*Z,Z,Z,Z*)-(eicosa-5,8,11,14-tetraenylthio)propionic acid **86** using 5-LO had a retention time of 10 minutes (hexane: isopropanol: acetic acid 980 : 20 : 1, flow rate 9 ml / min, semipreparative column).

Negative ion electrospray MS/MS, daughter ions of molecular anion: 393, 321, 303, 203, 131, 117 as shown in Fig 5.11. Positive ion electrospray HRMS [$C_{22}H_{38}SO_3 + Na$]⁺ calculated 417.2439, found 417.2442.

The (*E,Z,Z*)-(6-Hydroxyoctadeca-7,9,12-trienyloxy)acetic acid **104** formed by oxidation of (*Z,Z,Z*)-(octadeca-6,9,12-trienyloxy)acetic acid **83** using 5-LO had a retention time of 21 minutes (hexane : isopropanol : acetic acid, 900 : 100 : 1, flow rate 1 ml / min)

Negative ion electrospray MS/MS, daughter ions of molecular anion: 337, 319, 173, 163, 75 as shown in Fig 5.12. Positive ion electrospray HRMS [$C_{20}H_{34}O_4 + Na$]⁺ calculated 361.2355, found 361.2342.

(*E,Z,Z*)-(9-Hydroxyoctadeca-10,12,15-trienyloxy)acetic acid **105** formed by oxidation of (*Z,Z,Z*)-(octadeca-9,12,15-trienyloxy)acetic acid **84** using 5-LO had a retention time of 13.8 minutes as shown in Fig 5.14 (hexane : isopropanol : acetic acid, 960 : 40 : 1, flow rate 1 ml / min)

Negative ion electrospray MS/MS, daughter ions of molecular anion: 337, 319, 279, 215, 121, 75 as shown in Fig 5.13. Positive ion electrospray HRMS [$C_{20}H_{34}O_4 + Na$]⁺ calculated 361.2355, found 361.2348.

(*E,Z,Z,Z*)-(5-Hydroxyeicosa-6,8,11,14-tetraenylthio)acetic acid **107** formed by oxidation of (*Z,Z,Z,Z*)-(eicosa-5,8,11,14-tetraenylthio)acetic acid **85** using 5-LO had a

retention time of 19 minutes (hexane: isopropanol : acetic acid, 960 : 40 : 1, flow rate 1 ml / min).

Negative ion electrospray MS/MS, daughter ions of molecular anion: 379, 361, 317, 203 as shown in Fig 5.17. Positive ion electrospray HRMS $[C_{22}H_{36}SO_3 + Na]^+$ calculated 403.2283, found 403.2279

N-(*Z,Z,E,Z*)-(11-Hydroxyeicosa-5,8,12,14-tetraenyl)glycine **108** formed by oxidation of *N*-(*Z,Z,Z,Z*)-(eicosa-5,8,11,14-tetraenyl)glycine **88** using 5-LO had a retention time of 28 minutes (hexane: isopropanol : acetic acid, 800 : 200 : 15, flow rate 1 ml / min).

Negative ion electrospray MS/MS, daughter ions of molecular anion: 376, 223, 74 as shown in Fig 5.18. Positive ion electrospray HRMS $[C_{22}H_{35}NO_4 + Na]^+$ calculated 400.2464, found 400.2452.

Experimental for Results and Discussion: Chapter 5

(E,Z,E)-(6,13-dihydroxyoctadeca-7,9,11-trienyloxy)acetic acid **109**

A 50 μ M solution of (*Z,Z,E*)-(13-hydroxyoctadeca-6,9,11-trienyloxy)acetic acid **96** was prepared by evaporating stock solution (15.5 μ l), resuspending substrate in buffer (2.45 ml, pH 6.0, 0.005% Tween[®] 20, 40 mM KH_2PO_4) and saturation of the solution with oxygen. A background scan of the solution was recorded (300-200 nm). Potato 5-LO (50 μ l) was added and scans were recorded 0.1, 1, 2, 4, 8, 16, 32 and 64 minutes after beginning the incubation. The product was reduced with sodium borohydride and extracted into dichloromethane following acidification of the buffer with hydrochloric acid. The organic solvent was evaporated and the residue was dissolved in the HPLC solvent (hexane: isopropanol : acetic acid, 900 : 100 : 1) and (*E,Z,E*)-(6,13-dihydroxyoctadeca-7,9,11-trienyloxy)acetic acid **109** was isolated by HPLC (flow rate 0.6 ml / min, detection at 270 nm) with a retention time of 23 minutes.

Negative ion electrospray MS/MS, daughter ions of molecular anion: 353, 335, 253, 235, 179, 173, 113, 79, 75 as shown in Fig 6.2. Positive ion electrospray HRMS $[\text{C}_{20}\text{H}_{34}\text{O}_5 + \text{Na}]^+$ calculated 377.2304, found 377.2303.

Spectroscopic analysis of the coincubation of arachidonic acid **3 and (Z,Z,E)-(13-hydroxyoctadeca-6,9,11-trienyloxy)acetic acid **96** with 5-LO**

In four quartz cuvettes (3 ml), solutions containing a final concentrations of 50 μM arachidonic acid **3** and 0, 2.5, 5 or 50 μM (Z,Z,E)-(13-hydroxyoctadeca-6,9,11-trienyloxy)acetic acid **96** in buffer (2.5 ml, 0.04 M KH_2PO_4 , pH 6.3) were prepared from stock solutions in dichloromethane. Potato 5-LO extract (50 μl) was added to each cuvette and the change in absorbance over time was recorded for 5 minutes. The results are shown in Fig 6.3 and the change in absorbance and maximum rate of change in absorbance are shown in Table 6.1.

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